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Characterisation of the equine macrophage / monocyte

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Declaration

The thesis presented is the work of the author except where stated otherwise by reference and/or acknowledgement. Any work presented, which has been conducted by (or in collaboration with) others is explicitly acknowledged. Any part of this work has not been submitted for award or degree at any other university.

Anna Eleonora Karagianni Edinburgh, June, 2014

Dedication

To the youngest member of my family, Leto – Sofia, who has brought to us so much happiness.

To my parents who have so generously offered me their love and support in every step of my life.

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List of relevant publications and presentations

Publications relevant to the thesis

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Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. The equine macrophage/monocyte. (*Manuscript in preparation*)

Karagianni, A. E., Kapetanovic, R., Summers K., McGorum, B. C., Hume, D. A. & Pirie, R. S. Comparative microarray analysis between AMs and PMs of the horse and gene expression analysis of LPS treated equine AMs – similarities with human AM. (*Manuscript in preparation*)

Karagianni, A. E., Kapetanovic, R., Summers K., McGorum, B. C., Hume, D. A. & Pirie, R. S. The effect of training on the basal gene expression of AMs derived from Standardbred racehorses. (*Manuscript in preparation*)

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. Equine exercise immunology – emphasis on the equine monocyte/macrophage. (*Manuscript in preparation*)

Publication relevant to the thesis but not part of it

Kapetanovic, R., Fairbairn L., L., Moffat, L., **Karagianni, A. E.**, Downing, A., Fleming, R. H., Archibald, A. L. & Hume, D. A. 2014. GSE52705: Phenotype heterogeneity and maturation of alveolar macrophages in the domestic pig. Gene Expression Omnibus.

Conference abstracts

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. 2013. Aug 2013. *Characterisation of the equine macrophage/monocyte – a step in understanding equine pulmonary immunity*. Proceedings of the 10th International Veterinary Immunology Symposium at Milan (p.196). (Oral presentation)

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. 2013.*Characterisation of the equine macrophage/monocyte*. Proceedings of the Research Student Day - Postgraduate Presentations at the Royal Institute and the Royal (Dick) School of Veterinary Studies (p.7). (Oral and poster presentation)

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. 2013. *Characterisation of the equine macrophage/monocyte*. Proceedings of the British Society of Animal Science and the Association of Veterinary Teaching and Research workshop. Nottingham (p. 112). (Oral presentation)

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. 2012. *Characterization of the equine alveolar macrophage – a first step in understanding inflammatory airway disease in the horse*. Proceedings of the 30th Annual Symposium of the Veterinary Comparative Respiratory Society, Columbia, Missouri (p 126). (Poster presentation)

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. 2012. *Characterisation of the equine macrophage-a first step in understanding inflammatory airway disease*. Proceedings of the 4th European Veterinary Immunology Workshop (EVIW) Edinburgh (p. 42). (Poster presentation)

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. 2012. *Characterisation of the equine macrophage/monocyte*. One days Seminar, Equine Section, Glasgow University Veterinary School. (Oral presentation)

Karagianni, A. E., Kapetanovic, R., McGorum, B. C., Hume, D. A. & Pirie, R. S. 2012. *Characterisation of the equine alveolar macrophage*. Proceedings of the 5th

European College of Equine Internal Medicine Congress, Edinburgh. (Poster presentation)

Abstract

Inflammatory airway disease (IAD) is a common performance limiting pulmonary disorder in young racehorses in training. Although the precise aetiopathogenesis is poorly understood, proposed mechanisms include opportunistic bacterial infections and/or suboptimal air-hygiene. Since alveolar macrophages (AMs) are the first line defence in the lungs of mammalian species, they may constitute an appropriate therapeutic target cell in the treatment and the prevention of opportunistic airway infections. This thesis aimed to investigate the basic biology of the equine AM. A series of experiments were conducted to investigate the function and phenotype of this cell and comparisons made with equine macrophages derived from other anatomical sites and macrophage datasets derived from other species. The lung environment is unique, and may direct a unique phenotype and function compared with macrophages derived from other sites. Macrophages were isolated from the lungs, peritoneal cavity and other regions of healthy horses. Excellent cell recovery was demonstrated and associated with good viability, RNA yield and a demonstrable response to several stimuli, both when fresh and following cryopreservation. AMs produced tumor necrosis factor alpha (TNFα) when stimulated with lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (Poly IC) and heat-killed *Salmonella typhimurium* and were actively phagocytic. By comparison, peritoneal macrophages (PMs) did not respond to these inducers and lacked phagocytic activity. In contrast to AMs, which showed high expression of the specific macrophage markers cluster of differentiation (CD) 14, CD163 and toll-like receptor 4 (TLR4), PMs lacked CD14. Moreover, gene expression analysis revealed an alternative macrophage activation for AMs, whereas PM showed a hybrid macrophage activation potentially attributed to the phenomenon of endotoxin tolerance.

The response of equine AMs to LPS was analysed using microarrays. There was significant change in the expression of 240 genes. Those that were upregulated included well known inflammatory genes such as *TNFα*, *IL1A* and *CXCL6*. The pattern of response more closely resembled human and pig macrophages than mouse, including the LPS-induced expression of *STAT4*, *IDO*, *IL7R* genes and the failure to produce nitrite in response to LPS. These data suggest that the horse may represent a

suitable animal model for human macrophage-associated lung inflammation, and conversely that data from humans may translate to horses.

A final aim of this study was to investigate the effect of exercise on equine AM function. Therefore, AMs were isolated from bronchoalveolar lavage samples obtained from Standardbred racehorses at rest and during the training period and microarray analysis performed. Despite important limitations of the study, a few mechanisms at the molecular level were detected which may be involved in the development of either training-associated symptoms of, or susceptibility to IAD. Overall, this thesis aims to improve our understanding of equine macrophage biology and to provide useful information regarding the role of AMs in exercise-associated inflammation. Moreover, the findings presented here may help to inform future preventative pharmacological and/or managemental interventions for IAD.

Abbreviations

ACTH: adrenocorticotropic hormone

AEC: alveolar epithelial cell

AM: alveolar macrophage

ANS: autonomic nervous system

ATP: adenosine triphosphate

BALF: bronchoalveolar lavage fluid

BMDM: bone marrow derived monocyte

Btk: Bruton's tyrosine kinase

CAGE: cap analysis gene expression

CRH: corticotropin releasing hormone

CpG-ODN: cytosine phosphate guanine oligodeoxynucleotide

CSF: colony stimulating factor

CSF1R: colony stimulating factor 1 receptor

CXCL: chemokine (C-X-C motif) ligand

DAVID: database for annotation, visualisation, and integrated discovery

DC: dendritic cell

DCC: differential cell count

dsRNA: double stranded ribonucleic acid

e-CAS: equine macrophage cell line

EIPH: exercise-induced pulmonary haemorrhage

ELISA: enzyme linked immunosorbent assay

ET: endotoxin tolerance

FDR: false discovery rate

FLT3L: FMS-like tyrosine kinase 3 ligand

G-CSF: granulocyte- colony stimulating factor

GH: growth hormone

GM-CFU: granulocyte macrophage colony forming unit

GM-CSF: granulocyte macrophage- colony stimulating factor

GPI: glycosylphosphatidylinositol

HPA: hypothalamic-pituitary-adrenal axis

HSC: haematopoietic stem cell

IFNα: interferon alpha

IFNγ: interferon gamma

Ig: immunoglobulin

IL: interleukin

IM: interstitial macrophage

iNOS: inducible nitric oxide synthase

IP10: interferon gamma-induced protein 10

iPPOV: Inactivated parapoxvirus ovis

IRF: interferon regulated factors

i.v: intravenous

NK: natural killer

LAK: lymphocyte activated killer

LBP: lipopolysaccharide-binding protein

LPS: lipopolysaccharide

M-CFU: macrophage colony forming unit

MCL: markov cluster algorithm

M-CSF : macrophage- colony stimulating factor

MD2: myeloid differentiation protein-2

MHC: major histocompatibility complex

MIP2: macrophage inflammatory protein 2

MMP: matrix metalloproteinase

MPC: Mononuclear phagocyte

MPS: mononuclear phagocyte system

MyD88: myeloid differentiation primary response gene 88

NO: Nitric oxide

NK: natural killer

PAMP: pathogen-associated molecular pattern

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PCA: principal-component analysis

PGE: prostaglandin E

PIM: pulmonary intravascular macrophage

PLF: peritoneal lavage fluid

PM: peritoneal macrophage

PMN: polymorphonuclear

Poly IC: polyinosinic polycytidylic acid

R: correlation coefficient

RANKL: receptor activator of nuclear factor kappa-B ligand

RANTES: regulated on activation, normal T cell expressed and secreted

RAO: recurrent airway obstruction

RBC: red blood cell

Rh: recombinant human

RPL32: 60S ribosomal protein L32

RT qPCR: real time quantitative polymerase chain reaction

SiglecF: sialic acid-binding immunoglobulin-like lectin F

SOCS: suppressors of cytokine signaling

SP: surfactant protein

STAT: signal transducers and activators of transcription

sTNFR: soluble tumor necrosis factor receptor

TACE: tumor necrosis factor converting enzyme

TGFβ: transforming growth factor beta

TLR: toll like receptor

TNFα: tumor necrosis factor alpha

TRAF: TNF receptor-associated factor

TRIF: TIR-domain-containing adapter-inducing interferon-β

URTI: upper respiratory tract infections

VEGFA: vascular endothelial growth factor alpha

Chapter 1: Introduction

1.1 Macrophage biology and the mononuclear phagocyte system

Macrophages (from Greek "μακρόν" meaning big and "φαγείν" meaning to eat) are large leukocytes (white blood cells) that comprise 10-15% of all cells in most organs of all animal species (Hume et al., 2002, Gordon and Taylor, 2005, Hume, 2008a). They were originally identified in the 19th century by Metchnikoff, who distinguished them from related microphages, now known more commonly as granulocytes or polymorphonuclear (PMN) leukocytes. They are mononuclear, in contrast with the apparent multinuclearity of PMN cells (Kaufmann, 2008). Their numbers increase in tissues through extravasation and/or local proliferation in response to inflammation, injury and malignancy and they play a crucial role in tissue remodelling and the innate and adaptive immune system (Hume, 2006).

Macrophage functions include antigen presentation, phagocytosis, cytokine production, microbicidal activity, tissue repair and the general regulation of tissue homeostasis **(Figure 1.1)**. The population of macrophages and their progenitors in the whole body defines the mononuclear phagocyte system (MPS) (Volkman and Gowans, 1965, Hume et al., 2002, Hume, 2008b). The MPS of adults consists of cells originating from bone marrow progenitor cells that become monocytes circulating in the blood. Monocytes may enter tissues during inflammation and may differentiate into tissue resident phagocytes (Hume et al., 2002). Cells of the MPS may present in any tissue type (Gordon and Taylor, 2005) (http://www.macrophages.com/category/imagelibrary/macrophage-images/tissue-macrophages). **Table 1.1** lists the main cells that comprise the MPS in adults.

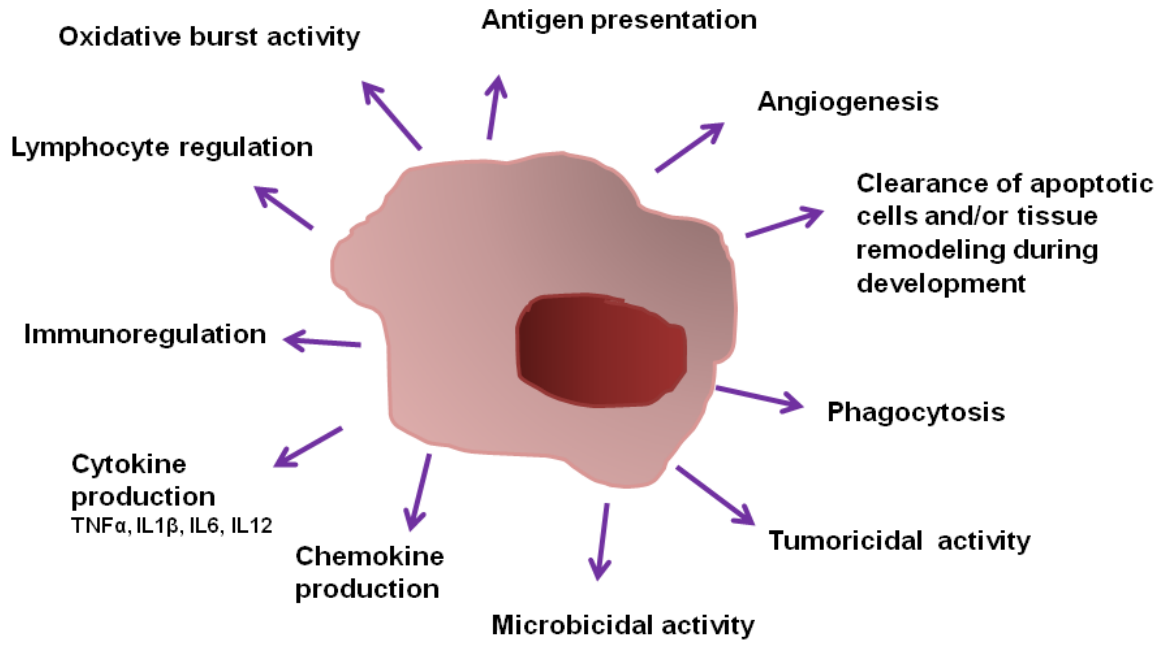


Figure 1.1: Summary of macrophage functions.

Mononuclear phagocytes	***Anatomical site***
Osteoclasts	Bone matrix
Monocytes	Bone marrow, blood, can migrate to any tissues
Histiocytes	Connective tissue
Macrophages of the central nervous system	Central nervous system
Microglial cells	*Parenchyma of the central nervous system*
Meningeal macrophages	*Meninges*
Choroid plexus macrophages	*Interface between the blood and the cerebrospinal fluid in the brain*
Lung macrophages	Lungs
Alveolar macrophages	*Airway wall of the pulmonary alveolus*
Pulmonary intravascular macrophages (not detected in healthy humans/mice, but present in horse, pig, sheep and other mammals)	*Capillary endothelium on the thicker side of the alveolar septum*
Interstitial macrophages	*Lung parenchyma*
Perivascular macrophages	Small blood vessels
Peritoneal macrophages	Peritoneal cavity
Intestinal macrophages	Lamina propria
Thymic macrophages	Thymus
Kupffer cells	*Liver, vascular sinuses, attached to the outer surface of endothelial cells*
Splenic macrophages	Spleen
Red pulp macrophages	*Spleen red pulp*
Marginal zone macrophages	*Spleen marginal zone*
White pulp macrophages	*Spleen white pulp*
Metallophilic macrophages	*Surround the splenic white pulp, adjacent to the marginal sinus*
Tingible body macrophages	*Spleen white pulp, germinal centres of lymph nodes*
Pleural macrophages	Pleural cavity
Dendritic cells (DCs)	Skin, inner lining of the nose, lungs, stomach, lamina propria, lymph nodes
Langerhans cells	*Epidermal layer of the skin*

Table 1.1: Main types of cells comprising the mononuclear phagocyte system in adult mammals.

Figure 1.2 shows a schematic diagram of macrophage development and differentiation in rodents (Gordon and Taylor, 2005). During development, murine macrophages appear first in the yolk sac, where they seem to arise through a distinct pathway that does not require the haemapoietic transcription factor, c-myb (Vanfurth, 1992, Schulz et al., 2012). Subsequently, definitive haematopoiesis in mammals arises in the aorta-gonad-mesonephros and then the liver, where the classical blood monocytes are produced, differentiate to macrophages and migrate throughout the embryo (Lichanska and Hume, 2000). Later in development, haematopoiesis commences within bone marrow and bone marrow derived monocytes (BMDMs) appear in the blood (Vanfurth, 1992, Lichanska and Hume, 2000).The granulocyte macrophage colony forming unit (GM-CFU) that derives from the haematopoietic stem cell (HSC) develops into the macrophage colony forming unit (M-CFU) and subsequently into the monoblast and the pro-monocyte, that are the progenitors of the monocyte/macrophage (Gordon and Taylor, 2005).

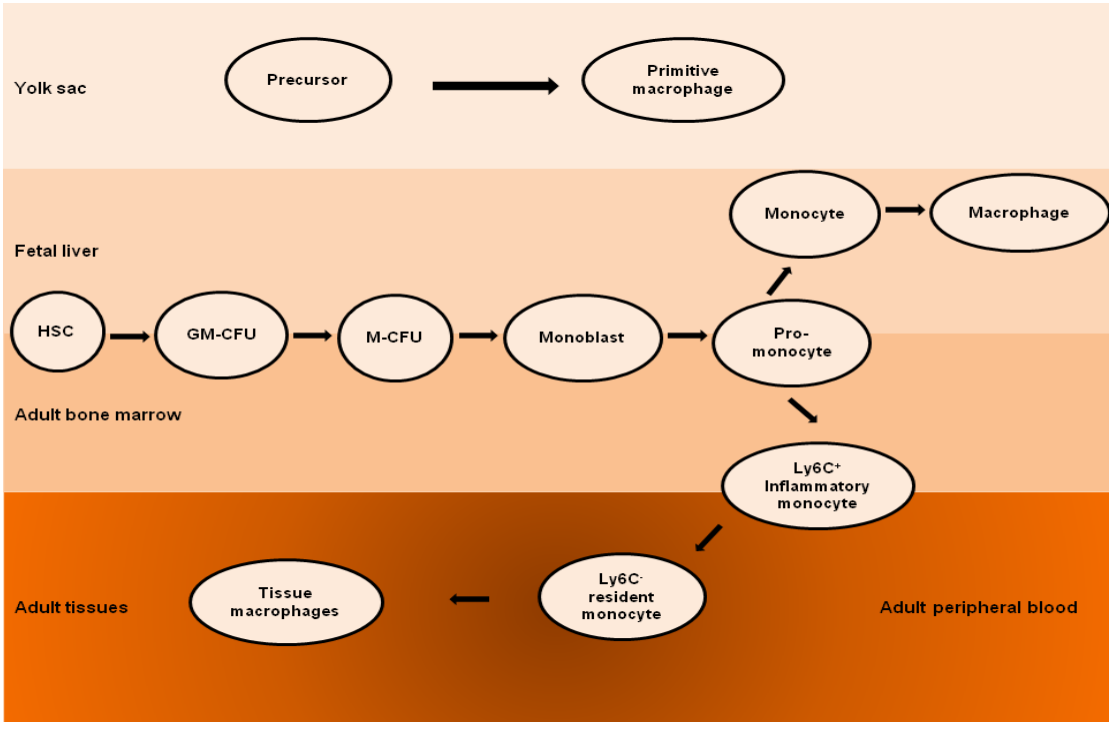


Figure 1.2 Origin of the murine mononuclear phagocyte system.

Figure adapted from Gordon et al (2005). HSC: haematopoietic stem cell, GM-CFU: granulocyte/macrophage colony-forming unit, M-CFU, macrophage colony-forming unit.

The concept that tissue macrophages are renewed from monocytes or adult haemopoietic progenitors in the steady state has recently been challenged (Merad et al., 2002, Ajami et al., 2007, Hashimoto et al., 2013). Lineage-trace studies suggest that brain, lung, splenic red pulp, peritoneal and bone marrow tissue macrophages in the mouse do not originate from monocytes (Schulz et al., 2012, Hashimoto et al., 2013, Yona et al., 2013). As an example, no macrophages are detected in the lungs of mice before birth. Rather they are derived from fetal monocytes and adopt their final mature phenotype post-natally (Guilliams et al., 2013). This maturation requires strong input from colony stimulating factor 2 (CSF2) and takes around seven days, with mature AMs appearing within the lungs 1-3 days following birth. Afterwards they are self-maintained in the lung milieu throughout life and do not require a significant degree of blood monocyte differentiation (Guilliams et al., 2013).

1.1.1 Important factors of haemopoietic regulation

The proliferation and differentiation of blood cells is controlled by hormone-like regulators collectively called colony stimulating factors (CSFs) (Hamilton and Achuthan, 2013). These include macrophage-CSF (M-CSF, commonly known as CSF1), granulocyte macrophage-CSF (GM-CSF, commonly known as CSF2) and granulocyte-CSF (G-CSF, commonly known as CSF3), responsible for the differentiation and proliferation of stem cells into myeloid cell types (Bradley et al., 1967, Hamilton and Achuthan, 2013). The last is responsible for neutrophil production and proliferation and although difficult to detect in blood, is secreted to a greater extent during infectious or inflammatory conditions (Roberts, 2005, Hamilton, 2008). CSF2 promotes the differentiation, proliferation and activation of granulocytes, macrophages and dendritic cells (DCs) and can act as a proinflammatory cytokine (Hamilton, 2008); whereas CSF1 promotes differentiation proliferation and maintenance of the monocyte/macrophage lineage (Hamilton and Achuthan, 2013). In addition to its proinflammatory function, CSF1 also has anti-inflammatory activities (Hamilton, 2008). Its receptor, CSF1R (M-CSFR, CD115 or FMS), is expressed by all cells of the MPS **(Table 1.1)** and their progenitors and is

essential for the differentiation of HSCs into monocyte/macrophages (Cecchini et al., 1997).

CSF1 can interact with other factors, including CSF2, the FMS-like tyrosine kinase 3 ligand (FLT3L), interleukin 3 (IL3) and receptor activator of nuclear factor kappa-B ligand (RANKL) (Barton and Mayer, 1989, McKenna et al., 2000, Yao et al., 2002, D'Amico and Wu, 2003, Chitu and Stanley, 2006, Fleetwood et al., 2007, Bonifer and Hume, 2008, Hume, 2008b), resulting in the generation of specific subsets such as DCs or osteoclasts and the initiation of emergency haematopoiesis in response to pathogen challenge. CSF2 may have a specific function in the lung, since deficiency of this factor in humans and mice leads to alveolar proteinosis (Nishinakamura et al., 1995, Kitamura et al., 1999). The central importance of CSF1 is evident from mutations of either *Csf1* or *Csf1r* genes in rodents, which generates macrophage and osteoclast insufficiency and has severe pleiotropic effects on multiple organ systems (Chitu and Stanley, 2006, Hume, 2008b, Gow et al., 2010). Recently, IL34, an alternative CSF1R ligand, has been discovered (Lin et al., 2008), explaining the fact that a CSF1R mutant has a more severe phenotype than a CSF1 mutation (Dai et al, 2002). IL34 seems to have a non-redundant function in development of certain macrophage populations. IL34-deficient mice had reduced Langerhans and microglia cells and a compromised immune response to skin antigens and viral infection of the central nervous system (Marks and Lane, 1976, Wang et al., 2012).

Other factors such as IL6, the transcription factor PU.1 (responsible for CSF1R expression) and the cytokine stem cell factor, also contribute to monocyte/macrophage differentiation, although to a lesser extent (Chomarat et al., 2000, Hume et al., 2002, Gordon and Taylor, 2005, Pollard, 2009). Most knowledge of haemopoietic regulation derives from mouse and human studies. This project deals specifically with the MPS of the horse. There is limited information on the function of macrophage growth factors in this species. Sequence variation in mammalian *CSF1* and *IL34* sequences has been shown to influence species cross-reactivity (Gow et al., 2013). Equine *CSF1* has not yet been fully cloned and expressed. Equine *IL34*, on the other hand, has been sequenced, and the predicted protein is 75% conserved with human/primates (differing mainly in the final 10-13

C-terminal amino acids), 86% with cattle and 71% with mice. Recombinant human (rh) CSF1 is active on all species-derived cells previously tested (Garceau et al., 2010, Kapetanovic et al., 2012, Gow et al., 2013). Other groups have also successfully used rhCSF1 in addition to rhRANKL to grow equine osteoclasts from equine spongy bone and bone marrow cells (Perrotti et al., 2009, Gray et al., 2002).

Equine *CSF2* has been cloned and unlike the equivalent murine growth factor, was found to cross-react with human cells, consistent with the high level of sequence conservation (83.7% of nucleotides) (Vecchione et al., 2002, Steinbach et al., 2005). Interestingly, equine CSF2 protein differs from the equivalent factor in other mammals through an altered signal peptide, which generates a distinct N-terminus, and a frame-shift and alternative stop codon at the 3' end of the messenger ribonucleic acid (mRNA), which adds eight C-terminal amino acids (Mauel et al., 2006). While equine CSF2 was active on a human factor-dependent cell line (a cell line entirely dependent on the continuous presence of one or more of growth factors), a much higher concentration of the human factor was required to stimulate equine cells, indicating the effects of subtle sequence variations on receptor affinity (Hammond et al., 1999b, Vecchione et al., 2002). Equine CSF2 in combination with IL4 has also been used to drive equine monocyte differentiation towards DCs (Mauel et al., 2006).

1.2 Markers of the mononuclear phagocyte system

Mononuclear phagocytes (MPCs) share stellate morphology and characteristic location in tissue (Hume, 2006). They secrete certain enzymes (nonspecific esterase, lysosomal hydrolases, ectoenzymes) which can be identified by histochemical assays and possess numerous endocytic receptors, especially for the Fc portion of immunoglobulin and complement coated particles (Hume, 2006). Monoclonal antibody technologies, which have significantly improved cell surface protein identification, have been useful in defining mononuclear phagocytes, yet have also illustrated the remarkable phenotypic diversity of these cells (Hume et al., 2002,

Taylor et al., 2005, Hume, 2008a). This diversity is a significant focus of this project, which examines the phenotypic specialisation of macrophages in different tissues of the horse. The majority of published work on macrophage markers has studied mice and humans **(Table 1.2)**. This section will focus on what is known about markers in these species, before reviewing in detail what is known about the equine system.

Full name	***Official gene symbol***	***Synonyms***
Chemokine (C-C motif) receptor 1	CCR1	CD191, CKR-1, MIP1aR
Chemokine (C-C motif) receptor 2	CCR2	CC-CKR-2, CD192, CKR2, FLJ78302, MCP-1-R
Chemokine (C-X3-C motif) receptor 1	CX3CR1	CCRL1, CMKDR1, V28
Cluster of differentiation 14	CD14	_
Cluster of differentiation 163	CD163	M130, MM130
Fc fragment of IgG, low affinity III (a and b), receptor	FCGR3A and FCGR3B	CD16 (CD16a and CD16b)
Colony stimulating factor 1 receptor	CSF1R	C-FMS, CD115, CSFR
Epidermal growth factor -like module containing, mucin-like, hormone receptor-like 1	EMR1	F4/80
Integrin, alpha M (complement component 3 receptor 3 subunit)	ITGAM	CD11b, MAC-1
Integrin, alpha X (complement component 3 receptor 4 subunit)	ITGAX	CD11c
lymphocyte antigen 6 complex	Ly6C	_
Sialophorin	SPN	CD43, GPL115, "leukosialin", LSN
Signal-regulatory protein alpha	SIRPA	CD172α, BIT, MFR, MYD-1, P84, SHPS-1, SHPS1, SIRP, SIRP-ALPHA-1, SIRPalpha, SIRPalpha2

Table 1.2: Main monocyte/macrophage markers studied in mice and human.

Information for this table was based on the Hugo gene nomenclature committee (http://www.genenames.org/)

Numerous cell surface markers, including EMR1, CSF1R, CD14, CD16, CD11b, CD11c, Ly6C and several chemokine receptors (CCR1,CCR2, CX3CR1) have been used to categorise MPCs in blood and tissues into functional subgroups in mammals (Gordon and Taylor, 2005, Hume, 2006). Nevertheless, not all markers are consistently expressed on the same mononuclear phagocyte subtypes in different species, nor do they even exist in some cases (Gordon and Taylor, 2005).

For instance, EMR1, part of the EGF-TM7 family which also includes CD97 (Gordon et al., 1986) and considered a pan-macrophage marker, is encoded by the *EMR1* gene. Mice have only two EMR members (*EMR1* and *4*) while humans have four (*EMR1-4*) (Kwakkenbos et al., 2004). Human monocytes express EMR1-3, while EMR3 is expressed in granulocytes (Kwakkenbos et al., 2004, Matmati et al., 2007). It is likely that the human *EMR4* gene mutated to a pseudogene and became inactive after *Pan-Homo* divergence (Hamann et al., 2003). In mice, EMR1 and EMR4 are also well studied (Stacey et al., 2002, Schaller et al., 2002). Schaller *et al* (2002) demonstrated that *EMR1* deficient mice failed to show any functional abnormality, perhaps because another member of the EGF-TM7 family, such as EMR4, might compensate for the deficiency (Schaller et al., 2002, Stacey et al., 2002). Nevertheless, in a later study *EMR1* knockout mice showed a significant deficiency in the differentiation of regulatory T (Treg) cells, which highlighted the role of EMR1 in immunological tolerance (Lin et al., 2005).

The CD16, is highly expressed on subsets of human, porcine and equine monocytes (Ingersoll et al., 2010, Noronha et al., 2012, Fairbairn et al., 2013a). CD16 has been used as a marker in humans to distinguish subpopulations of blood monocytes (Passlick et al., 1989, Ziegler-Heitbrock, 2000, Ziegler-Heitbrock et al., 2010) but it has also been detected on natural killer (NK) cells and PMNs (Ravetch and Perussia, 1989). The *CD16* gene is duplicated (*CD16*a and *CD16b*) in the genome of most humans, but not in mouse, and one of the genes encodes a glycosylphosphatidylinositol (GPI) anchored form of the protein. CD16 is a low affinity Fc receptor that is shown to have a vital role in the inflammatory and immune responses of many species (Hazenbos et al., 1996, Galon et al., 1996). *CD16* deficient mice showed impaired function of immune cells and a defective

inflammatory response (Hazenbos et al., 1996) and copy number polymorphism of *CD16* is related to susceptibility to disease in both humans and rats (Aitman et al., 2006).

The most widely-used marker for the separation of monocytes from PBMCs is CD14. CD14 is the receptor for lipopolysaccharide (LPS) , lipopolysaccharide-binding protein (LBP) and toll like receptor 4 (TLR4) (Steinemann et al., 1994). It is a GPI anchored protein expressed highly on the cell surface of most monocytes and macrophages (Peterson et al., 1995, AntalSzalmas et al., 1997, Liu et al., 1998, Nanbo et al., 1999), although it is also detected to a lesser extent on human and murine neutrophils and B-cells (Chaby et al., 1984, Haziot et al., 1993, AntalSzalmas et al., 1997). Equine *CD14* has been cloned and sequenced, and the mRNA is expressed by horse monocytes (Kabithe et al., 2010). The coding sequence shares high homology with human *CD14* and the antihuman CD14 antibody was found to cross-react with equine monocytes in several studies (Steinbach et al., 2005, Durán et al., 2013).

Another important marker for the detection of MPS is CSF1R. Anti-CSF1R has been used to detect monocytes and macrophages in mice and humans (Hume and MacDonald, 2012), and a CSF1R-EGFP transgene provides a convenient marker in mice (Sasmono et al., 2003). Thus far, anti-CSF1R antibody is not available for the horse. Anti-pig CSF1R, produced in our group (*Manuscript submitted*), and anti-human CD115 (*unpublished*) apparently did not cross-react.

CD163 is a haemoglobin scavenger receptor that plays a role in the endocytosis of human haptoglobin–haemoglobin complexes and is specifically expressed on monocytes and tissue macrophages (Kristiansen et al., 2001). Moreover, it acts as an erythroblast adhesion receptor (Fabriek et al., 2007), a receptor for the TNF-like weak inducer of apoptosis (Bover et al., 2007) and as a gram positive and gram negative bacterial sensor (Fabriek et al., 2009). Apart from membrane bound CD163, it also exists in a soluble form (Moller et al., 2002). In man, CD163 is most highly-expressed in the $CD14^{++}$ classical monocytes, but CSF1 augments the expression of CD163 in all monocyte subsets (Tippett et al., 2011). Mouse monocyte

subsets $Ly6C^{++}$ and $Ly6C^{+}$ seem not to differentially express CD163 (Ingersoll et al., 2010), while in the rat CD163 is expressed in tissue macrophages but not in monocytes (Polfliet et al., 2006). Similar to humans, by using antihuman CD163 monoclonal antibody, it was shown that both equine monocytes and tissue macrophages express CD163 (Yamate et al., 2000, Steinbach et al., 2005). CD163 has a vital role in the modulation of the immune response and is considered to be a marker of anti-inflammatory macrophage activation (Komohara et al., 2006). Numerous factors have been reported so far to regulate its cell surface expression (Kowal et al., 2011). The anti-inflammatory factors IL10 and glucorticoids (Sulahian et al., 2000), the pro and anti-inflammatory cytokine IL6 (Weaver et al., 2007) and CSF1 enhance CD163 expression (Tippett et al., 2011), whereas other proinflammatory [tumor necrosis factor alpha (TNFα), IL1, interferon gamma (IFNγ)] and anti-inflammatory mediators [IL4, IL13, transforming growth factor beta (TGFβ)], as well as other factors (LPS, hypoxia) downregulate its expression (Weaver et al., 2007, Kowal et al., 2011). It is detected in several pathologic tissues and macrophages in humans and other species (Yamate et al., 2000, Tippett et al., 2011). In horses, CD163 was associated with wound healing and tissue repair (Lefebvre-Lavoie et al., 2005) and it was also detected in laminar tissue of horses with laminitis (Faleiros et al., 2011) and in intestinal macrophages from horses with large colon ischaemia (Grosche et al., 2011).

1.3 Monocyte subsets

Monocytes are commonly distinguished from other mononuclear cells (lymphocytes and natural killer cells) based upon the presence of the surface marker CD14, but they vary significantly in size, granularity and nuclear morphology. As noted above, monocytes can also be subdivided into distinct subclasses based upon additional markers. The available markers differ between species. CD16 antibodies, were found to divide human monocytes into two subsets by flow cytometry; $CD14^{+}CD16^{-}$ cells and $CD14^{+}CD16^{+}$ cells (Passlick et al., 1989). In addition to their phenotypic differences, these subsets had functional differences, characterised by different gene

expression profiles (Ingersoll et al., 2010, Passlick et al., 1989). The subsets differ in their level of CD14 expression, and subsequent analysis defined a third subset (Ziegler-Heitbrock et al., 2010). This subset, termed $CD14^{low}CD16^{high}$, consists of a small population which is enhanced under cytokine stimulation and inflammatory conditions (Ellery et al., 2007, Ancuta et al., 2003, Weiner et al., 1994). The International Union of Immunological Societies and the World Health Organization adopted a nomenclature in which the three blood monocyte subsets were defined as the classical (inflammatory) $CD14^{++}CD16^{-}$, the intermediate $CD14^{++}CD16^{+}$ and the nonclassical $CD14^{+}CD16^{++}$ (Ziegler-Heitbrock et al., 2010). This distinction is clearly somewhat arbitrary, and there is no absolute delineation on a fluorescence activated cell sorting between "low" and "high" for any of the markers (Ziegler-Heitbrock et al., 2010). A developmental relationship is believed to exist between the different monocyte subsets; the intermediate subset arises from classical monocytes in response to CSF1 or during infection (Weiner et al., 1994, Ziegler-Heitbrock et al., 2010). The populations have different functions and gene expression profiles and respond differently to infections **(Table 1.3)** (Geissmann et al., 2010, Wong et al., 2012, Wong et al., 2011). A recent detailed promoter-based analysis indicates that the large majority of "markers" do actually exhibit a gradient of expression, either increasing or decreasing in parallel with the variation in CD14 and CD16, strongly supporting the view that subsets are no more than a differentiation series (Schmidl et al., 2014).

Human Monocyte subset	Monocyte percentage	Main functions
Classical (inflammatory) $CD14^{++}CD16^{-}$	85 %	Phagocytosis
Intermediate $CD14^{++}CD16^{+}$	5%	T cell activation Reactive oxygen species production Angiogenesis Antigen presentation
Nonclassical $CD14^{+}CD16^{++}$	10%	T cell activation

Table 1.3: The human monocyte subsets nomenclature and their main functions.

Murine monocytes have also been subdivided into three different subpopulations that adopt the same nomenclature as human monocytes (classical, intermediate and non-classical), and have similar functions (Ziegler-Heitbrock et al., 2010). Moreover, the same developmental relationship seems to exist between the murine monocyte subsets, since the nonclassical subset is apparently derived from the classical subset (Sunderkötter et al., 2004). Unlike humans and horses, mice do not have duplicated *CD16* gene, CD16 is not used as a marker and CD14 is not as highly-regulated (Ziegler-Heitbrock et al., 2010). The most widely-used marker is Ly6C, although other markers, such as CD43 and the chemokine receptors CCR2 and CX3CR1, have also been used (Ziegler-Heitbrock et al., 2010). Monocytes with high Ly6C expression and low CD43 are considered as classical ($Ly6C^{++}CD43^{+}$), those with high expression of both cell markers are considered as intermediate ($Ly6C^{++}CD43^{++}$) and those with low Ly6C levels and high CD43 are considered as nonclassical ($Ly6C^{+}CD43^{++}$). Murine monocytes with high Ly6C expression share many similarities in their gene expression profile with human $CD14^{+}$ monocytes (Ingersoll et al., 2010), while those with low Ly6C expression were initially thought to contribute to tissue macrophage recruitment (Auffray et al., 2007, Auffray et al., 2009), but more recently have been considered as an end-stage blood macrophage that "patrols" the vessel wall (Auffray et al., 2007, Auffray et al., 2009, Yona et al., 2013).

In the pig, blood monocytes have been also subdivided into two main subpopulations based on their expression of CD14, CD16, CD163 and CD172α (Fairbairn et al., 2011, Fairbairn et al., 2013a). These consist primarily of $CD14^{++}CD163^{+}$ and $CD14^{+}CD163^{++}$ subpopulations in approximately equal numbers (Fairbairn et al., 2013a). Microarray analysis of these two subpopulations followed by comparisons with human and murine monocyte subpopulations suggested that there was no clear equivalent to the "nonclassical" cells, and the $CD14^{+}CD163^{++}$ cells were more "intermediate" in their phenotype (Fairbairn et al., 2013a) **(Table 1.4)**.

Human	*Mouse*	*Pig*
Classical (inflammatory) $CD14^{++}CD16^{-}$	$Ly6C^{++}CD43^{+}$	$CD14^{++}CD163^{+}$
Intermediate $CD14^{++}CD16^{+}$	$Ly6C^{++}CD43^{++}$	$CD14^{+}CD163^{++}$
Nonclassical $CD14^{+}CD16^{++}$	$Ly6C^{+}CD43^{++}$	Not detected

Table 1.4: The monocyte subset nomenclature of human, mouse and pig and their association.

There has been limited analysis of equine monocytes, but variation in CD14 and CD16 expression amongst monocyte "subsets" has been reported (Noronha et al., 2012). As early as 1976, horse monocytes were found to be a more appropriate source of indicator cells for human migration-inhibiting factor assays than guinea pig monocytes, which were previously the population of choice (Friedrich et al., 1976). A recent study divided equine monocyte-derived DCs into two different subpopulations; the immature DCs and the mature DCs (Moyo et al., 2013). In this study, equine monocytes differentiated into immature DCs via CSF2 and IL4 stimulation and into mature DCs via stimulation with a cytokine cocktail [TNFα, IL6, IL1β, prostaglandin E_2 (PGE_2) and IFNγ] (Moyo et al., 2013, Durán et al., 2013). Subsequent microarray analysis confirmed a clear distinction between these two cell subsets (Moyo et al., 2013). In contrast to human DCs, the mature cells did not express high levels of the traditional DC markers CD83 and CD206 (Moyo et al., 2013).

1.4 Macrophage activation

Macrophages, as an integral part of the MPS, detect and attempt to destroy potential pathogens, while alerting other components of the immune system (Kapetanovic and Cavaillon, 2007). Almost by definition, a successful pathogen evades macrophage-

mediated killing. Many pathogens do so by replicating within the phagocytes, despite being internalised (Friedrich et al., 2012). Macrophage recognition of pathogens is dependent on their expression of a range of cell surface and intracellular receptors, including the toll-like receptor (TLRs) and nucleotide oligomerisation domain classes (Taylor et al., 2005, Brooks et al., 2011, Akira and Sato, 2003). These receptors distinguish "non-self" by binding to molecules that are conserved amongst broad classes of pathogens but are absent in the host, the so called pathogen-associated molecular patterns (PAMPs). Furthermore, innate immune cells recognise molecules found in the extracellular environment in response to injury, the damage-associated molecular patterns (Tang et al., 2012).

The well characterised PAMPs include the following: LPS, a structural component of gram negative bacterial cell walls, detected by TLR4 and co-receptors CD14 and myeloid differentiation protein-2 (MD2); microbial lipoproteins, recognized by heterodimers of TLR2-TLR1 or TLR2-TLR6; single or double stranded RNA, recognised byTLR3 and RIGI; flagellin, recognised by TLR5; unmethylated DNA, recognised by TLR9 (Akira and Sato, 2003, O'Neill, 2006). In response to PAMP-mediated pathogen recognition, macrophages and other cells initiate a complex signalling cascade that leads to secretion of numerous effector molecules that in turn promote recruitment and activation of other cells to the process of inflammation (Hume, 2008b, Akira and Sato, 2003). This type of macrophage activation, mediated via TLRs and the T cell cytokine IFNγ, is widely known as classical activation (Mantovani et al., 2004). It is characterised by enhanced microbicidal-tumoricidal activity and proinflammatory cytokine (TNFα, IL1, IL12) and mediator (CXCL9, CXCL10, C-C chemokine receptor type 7) release (Mosser and Edwards, 2010, Mantovani et al., 2004).

An alternative activation phenotype is linked predominantly to the actions of the cytokines IL4 and IL13 (Mantovani et al., 2004, Gordon and Martinez, 2010). Alternative activation of macrophages is associated with distinct physiologic and pathologic conditions, such as wound healing, fibrosis, malignancy, hypersensitivity, parasitic infection and even homeostasis (Gordon and Martinez, 2010, Chawla, 2010, Mantovani et al., 2013). Based upon the relationship to the Th1 and Th2 states of T

helper cell function, these macrophage states have been referred to as M1 and M2, respectively, summarised in **(Figure 1.3)** (Gordon and Martinez, 2010, Mantovani et al., 2013). However, others have argued that the macrophage activation states are considerably more variable, and reflect the massive diversity of possible stimuli (Mosser and Edwards, 2010). Moreover, global meta-analysis of mouse and human microarray datasets do not support the idea of any transcriptional signature that is linked to the M1 or M2 states (Wells et al., 2003, Mabbott et al., 2010). Macrophages are characterised by a high level of plasticity and their regulation depends on different environmental challenges (Mosser and Edwards, 2010). Due to the large variety of *in vivo* environmental stimuli that macrophages interact with, several hybrid macrophage activations states may occur, characterised with an overlapping sate between M1 and M2 polarisation. Examples represent tumor associated macrophages (Mosser and Edwards, 2010, Biswas and Mantovani, 2010), endotoxin tolerant macrophages (Rajaiah et al., 2013, Pena et al., 2011), intestinal macrophages (Bain and Mowat, 2011, Mowat, 2011) and Kupffer cells (Hafenrichter et al., 1994).

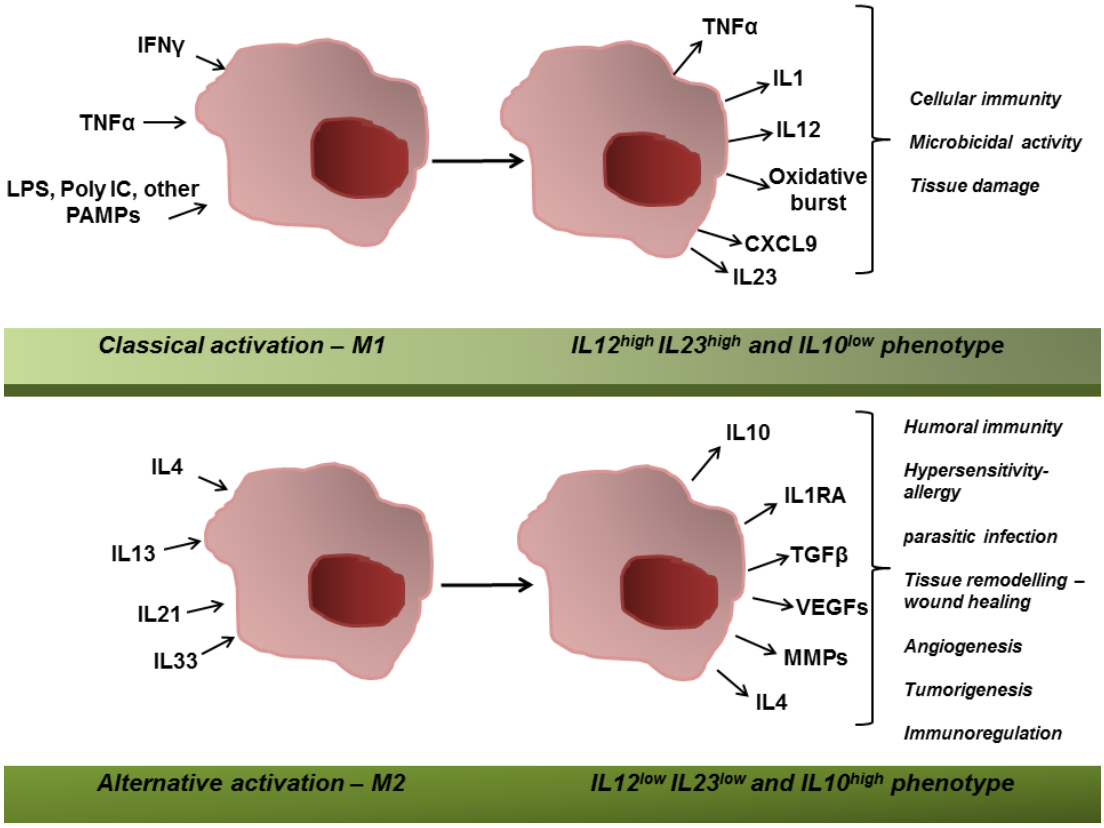


Figure 1.3: Classical and alternative activation of macrophages.

Classical macrophage activation (M1) is induced by TNFα, LPS or other PAMPs and results in a Th1 immune response, associated with microbicidal activity, inflammation and tissue damage. Alternative macrophage activation (M2) is induced by IL4, IL13, IL21 or IL33 and results in Th2 cytokine production, TGFβ, vascular endothelial growth factors (VEGFs) or matrix metalloproteinases (MMPs) secretion. The latter is related to humoral immunity, hypersensitivity, parasitic infections, tissue remodelling and repair, angiogenesis, immunoregulation and tumorigenesis.

In contrast to the extensive literature on humans, mice and even pigs (Fairbairn et al., 2011), there is very limited literature regarding the MPS of the horse, especially at a transcriptional level. This thesis constitutes a first step in the investigation of the MPS of the horse through the study of the alveolar and peritoneal macrophage (PM) response to several stimuli, in particular LPS. It also focuses on the effect of the microenvironment on the phenotype and function of different types of equine

macrophages, a subject area on which there is a dearth of information compared with the human and mouse literature.

1.4.1 The phenomenon of endotoxin tolerance

As discussed earlier **(Section 1.4)**, innate immune cells respond to pathogen-associated molecules via pattern recognition receptors, such as TLR4 in the case of LPS, and induce inflammation (Steinemann et al., 1994, Lien and Ingalls, 2002). Continuous exposure to LPS can result in the extreme production of proinflammatory cytokines, such as TNFα, IL1 and IL6, in turn leading to tissue damage, septic shock and ultimately death (Hotchkiss and Karl, 2003, Karin et al., 2006). A natural feedback mechanism, referred to as endotoxin tolerance, provides some protection against such an occurrence *in vivo*, thus limiting endotoxin-induced pathology. Macrophages/monocytes are critical in endotoxin tolerance as summarised in **Figure 1.4**.

Endotoxin tolerance is manifest in isolated macrophages. Tolerised mouse or human macrophages show a downregulation of several inflammatory cytokines such as TNFα, IL6, IL1β and an upregulation of anti-inflammatory cytokines such as IL10 and TGFβ, as well as of scavenger and c-type lectin receptors, negative regulators and antimicrobial genes (del Fresno et al., 2009, Mages et al., 2008, Biswas and Lopez-Collazo, 2009), in some ways resembling the so-called alternative macrophage activation or M2 phenotype (Deepak et al., 2007). Although antigen presentation activity of LPS tolerant macrophages is impaired, phagocytic activity remains high (del Fresno et al., 2009). These characteristics were also observed in monocytes derived from patients with sepsis (del Fresno et al., 2009, Pachot et al., 2006). Mouse macrophages and human monocytes become tolerised when exposed to LPS (del Fresno et al., 2009, Dobrovolskaia and Vogel, 2002). Del Fresno *et al* (2009) reported that a eight hour exposure of human monocytes to LPS is sufficient for them to become refractory to the effects of endotoxin for as long as five days (del Fresno et al., 2009). These results suggest the potential existence of a type of "memory'' in the innate immune system that could be modulated by epigenetic

factors (Biswas and Lopez-Collazo, 2009). Several *in vivo* and *in vitro* studies have been performed in order to mimic the optimal *in vivo* septic conditions in humans and study the endotoxin tolerance of macrophages/monocytes (Biswas and Lopez-Collazo, 2009, Dobrovolskaia and Vogel, 2002, del Fresno et al., 2009, Mages et al., 2008). It is suggested that the model of two consecutive endotoxin challenges, including a time lapse between them, represents the most appropriate model for studying endotoxin tolerance on macrophages/monocytes (Biswas and Lopez-Collazo, 2009). Thus, the two consecutive challenges are representative of a primary and secondary infection that may occur in human patients.

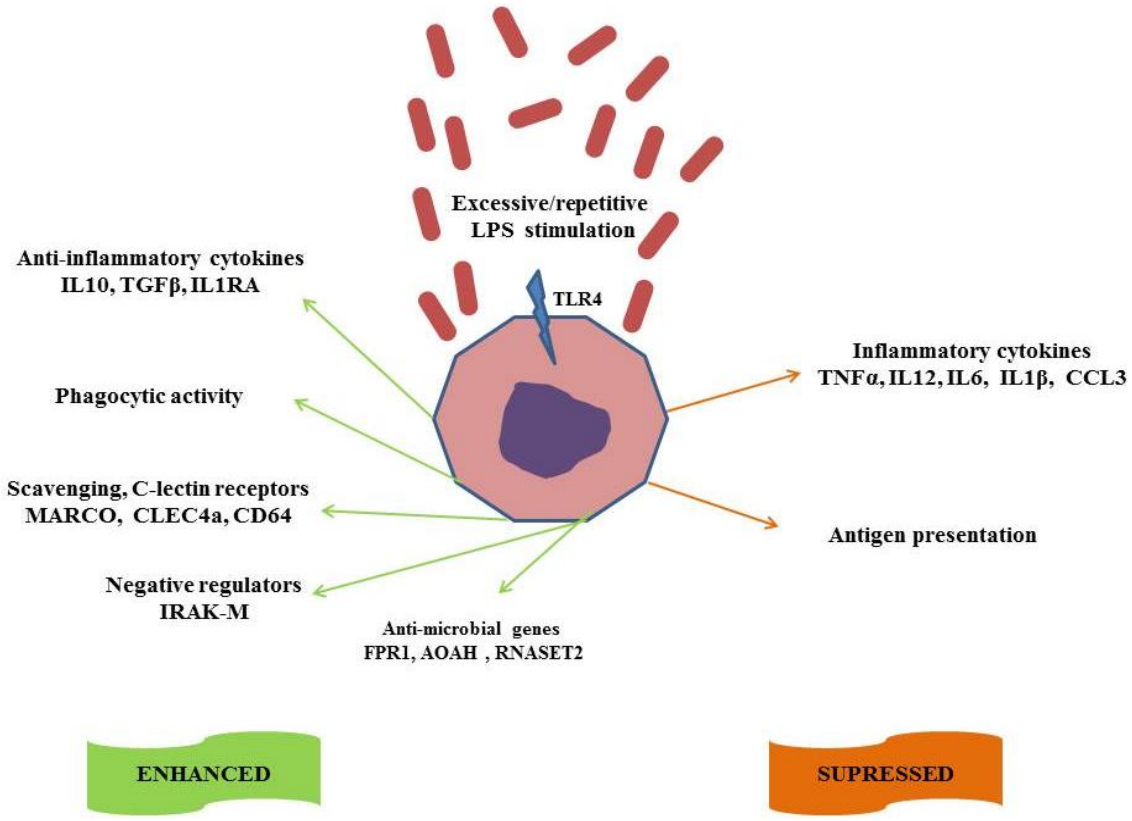


Figure 1.4: Function and phenotype of the endotoxin tolerant macrophage/monocyte.

After LPS restimulation with the same LPS dose, macrophages become refractory to LPS and overexpress anti-inflammatory cytokines, scavenging c-type lectin receptors and antimicrobial genes. They show low expression of inflammatory cytokines and have reduced antigen presentation even though their phagocytic activity remains high. MARCO: Macrophage receptor with collagenous structure, CLEC4a: C-type Lectin domain family 4, member A, FPR1: Formyl peptide receptor 1, AOAH: Acyloxyacyl hydrolase, RNASET2: Ribonucelase T2.

1.5 Lung macrophages and their microenvironment

1.5.1 Lung microenvironment

The lung is responsible for gas exchange and the alveoli provide an exceptionally large epithelial surface area to facilitate this process. For this reason, the lung is exposed to a great variety of aeroallergens and pathogens. Innate immunity provides the first line of defence against pathogens and also interacts with the adaptive immune system. It consists of structural defenses, antibacterial substances produced in the airways and phagocytosis (Coonrod, 1986).

Coughing, sneezing, nasal hair, the anatomic nature of the nasopharynx, glottis and trachea followed by the branching of bronchi and bronchioles restrict inhaled particulates greater than 5μm in diameter from entering the lower airways (Mercer and Crapo, 1998). The airway surface also contains complex glycoproteins called mucins, which capture the inhaled particulates and transport them to the oropharynx where they are swallowed or expectorated. Particles less than 5μm in diameter (e.g. microorganisms) can enter the lower airways. Airway antimicrobial factors are produced from ciliated cells and goblet cells of the respiratory epithelium in response to these pathogens (Martin et al., 1992, Coonrod, 1986, Hiratsuka et al., 1998, Schnapp and Harris, 1998). Examples include lysozyme, complement, immunoglobulin (Ig) A and G, fibronectin, defensins, lactoferin and LBP (Coonrod, 1986, Martin et al., 1992, Hiratsuka et al., 1998, Schnapp and Harris, 1998). These substances either have direct anti-microbial activity or stimulate AMs and PMN leukocytes to initiate immune defence.

The lung alveoli consist of type I and type II alveolar epithelial cells (AECs). Type II AECs are more cuboidal, whereas type I AECs are thinner and both are self renewed and differentiated (Lambrecht, 2006). Lung alveoli are rich in capillaries, separated from the type I AECs only by a thin (0.2μm) membrane, thus facilitating efficient gas exchange (Lambrecht, 2006). During inhalation of microorganisms or toxic particles there is a high risk of these substances entering the blood. Therefore, in order to prevent such an occurrence, a network of immune cells are present to

preserve host defence at this level (Lambrecht, 2006). This network is comprised mainly of macrophages, but also includes DCs, AECs, lymphocytes and polymorphonuclear cells. AMs reside in the lung alveoli and are the first cells that contact inhaled antigens in the airways. AMs alone can cope with the instillation of up to 10^9 bacteria into the trachea before there is any requirement for the adaptive immune system to be recruited (Lambrecht, 2006).

It is important that immune activation is not triggered unnecessarily as inflammation and immune cell recruitment can lead to oedema and obstruction of the alveolar wall, thus compromising gas exchange. Indeed, given the airway surface area and the quantity of foreign particles inhaled on a breath-by-breath basis, it is perhaps surprising why the airways are not in a constant state of inflammation. Under normal conditions, AMs secrete small amounts of cytokines, have low phagocytic activity, low CD11b expression and limited interaction with the adaptive immune system and are characterised by an M2 phenotype (Holt, 1978, Jones et al., 2013). Antigen presentation and T cell activation are suppressed by the physical separation of antigen-presenting cells expressing class II major histocompatibility complex (MHC) from AMs, which are adherent to the alveolar wall (Holt et al., 1993), and via AM secretion of anti-inflammatory molecules such as TGFβ (Holt et al., 1993). AEC may express the integrin $a_v\beta_6$, which binds the TGFβ and activates it, thereby preventing AM activation (Takabayshi et al., 2006, Lambrecht, 2006). Nevertheless, during infection AMs are activated by TLRs and detach from the AECs which stop expressing $a_v\beta_6$ integrin (Lambrecht, 2006, Takabayshi et al., 2006). Thus AMs may be able to switch to become classically-activated macrophages by releasing proinflammatory cytokines, inducing phagocytosis and interacting with the adaptive immunity (Lambrecht, 2006, Takabayshi et al., 2006). This pathway is outlined in **Figure 1.5**.

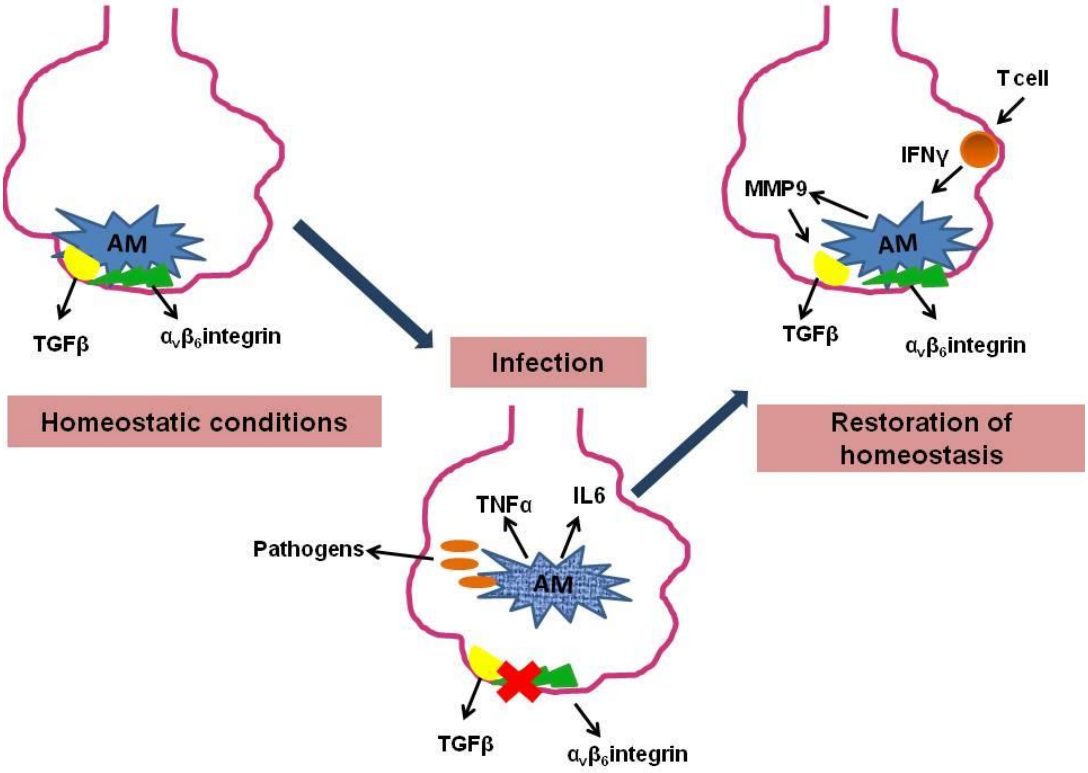


Figure 1.5: Mechanism of AM activation.

During homeostatic conditions AMs are attached to the AECs located on the surface of the alveoli, which express $a_v\beta_6$ integrin in a TGFβ dependent way and AM activity is suppressed. Under infection the expression of $a_v\beta_6$ integrin is lost and the inhibition of AMs by TGFβ is blocked; thus AMs are activated in order to kill the pathogens by phagocytosis and secretion of proinflammatory cytokines. A few days after infection, activated T cells trigger the production of the matrix metallopeptidase 9 (MMP9) by AMs. MMP9 production activates the TGFβ which inhibits AM activation and restores homeostasis.

1.5.2 Phenotype and function of alveolar macrophages in comparison with macrophages/monocytes from different anatomical locations in humans and mice

At least three types of macrophages have been identified so far in the lungs, the AMs, the interstitial macrophages (IMs), and the pulmonary intravascular macrophages (PIMs) that are absent in healthy humans and mice (Schneberger et al., 2012, Laskin et al., 2001). Published studies have differed as to whether there are

significant functional differences between these populations of lung macrophages (Bilyk and Holt, 1991, Nibbering et al., 1987, Cai et al., 2014, Bilyk et al., 1988). AMs are believed to originate from IMs, are bigger in size with more granules in their cytoplasm and they resemble resident tissue macrophages, whereas IMs resemble blood monocytes (Laskin et al., 2001). Despite the functional and morphological differences between these two cell types, both are essential for lung homeostasis and immune response (Laskin et al., 2001, Cai et al., 2014).

Compared to both blood monocytes and other tissue derived macrophages, a number of surface markers are specifically enriched in AMs of several species, (Bosio and Dow, 2005, Gonzalez-Juarrero and Orme, 2001, von Garnier et al., 2005). For example, in the mouse, AMs showed high expression of CD11c and DEC-205, while PMs did not express these two surface markers; rather, they expressed high levels of CD11b and EMR1 (Guth et al., 2009). Moreover, DEC-205 has also been found in AMs, but not in other types of macrophages (Bilyk and Holt, 1991, Bosio and Dow, 2005, Bosio et al., 2005). This phenotype seems to resemble that of DCs, which are characterised mainly by CD11c, DEC-205, MHC II, CD40, CD80 and CD86. Sialic acid-binding immunoglobulin-like lectin F (SiglecF), a single-pass transmembrane surface protein that triggers apoptosis in human eosinophils is also expressed by mouse AMs but not by PMs (Feng and Mao, 2012).

AMs express the integrin CD11b only when activated. During infection, $CD11b^{+}/CD11c^{-}$ monocytes migrate to the lungs, where expression of CD11c increases and the cells develop as $CD11b^{+/mild}/CD11c^{+/mild}$, with subsequent differentiation into AMs, IMs or DCs. Therefore, it is likely that $CD11b^{-}/CD11c^{+/high}$ AMs or immature DCs in the alveolar spaces are activated due to infection and increased CD11b expression, resulting in $CD11b^{+/high}/CD11c^{+/high}$ activated macrophages or differentiating DCs (Gonzalez-Juarrero et al., 2003).

The phenotype of lung macrophages may be partly dependent on the production of local cytokines, such as CSF1 and CSF2 (Chen et al., 1988, Higgins et al., 2008, Trapnell and Whitsett, 2002). Apart from CSF2, surfactant protein (SP) D contributes to macrophage differentiation in the airway microenvironment. SPA

downregulates the ability of bone marrow derived DCs to develop antigen presenting capacities and inhibits macrophage function in mouse or human lungs (Brinker et al., 2003, Gardai et al., 2003, Janssen et al., 2008, Guth et al., 2009). Therefore, the local microenvironment is of major importance for their growth and activity (Gonzalez-Juarrero et al., 2003).

There are contradictory views about the interactions between AMs and T cells. One view is that AMs have relatively poor antigen presentation activity, presumably avoiding excessive lung inflammation (Thepen et al., 1989). In humans, some studies claim that AMs are poor at inducing activation of naïve T cells, but active in eliciting a recall response. Others suggest that AMs are effective antigen presentation cells for initiation of T cell-mediated immunity (Lipscomb et al., 1986, Lyons et al., 1986, Toews et al., 1984, Upham et al., 1997, Weissler et al., 1994, Twigg et al., 1989, Nicod et al., 1987). In response to pathogen challenge, a subset of AMs are able to migrate to the lung lymph nodes, in particular at B cell sites, and transfer antigens (Kirby et al., 2009).

AMs also showed a high capacity for macropinocytosis, a feature shared with immature DCs and less so by mature tissue macrophages (Sallusto et al., 1995). Similar to DCs, the macropinocytosis of AMs was reduced after stimulation with LPS, while that of PMs remained unaffected (Sallusto et al., 1995). The same study also demonstrated AMs to have a greater antigen presenting capacity compared to PMs. So-called cross-presentation, the processing and presenting of antigens via MHC I to $CD8^+$ T cells, has been considered an exclusive function of DCs (Guth et al., 2009). Moreover, after 24h culture AMs showed greater stimulation of a mixed lymphocyte reaction than PMs (Guth et al., 2009). This level of T cell stimulation was comparable to that induced by bone marrow-derived DCs and blood monocyte-derived DCs (Sallusto et al., 1995). Hence, the consensus is that in some respects AMs resemble immature DCs (Guth et al., 2009). On the other hand, AMs can also have immunosuppressive effects. A least *in vitro*, AMs are able to inhibit mitogen induced proliferation, suppress mixed lymphocyte reactions and restrain tumoricidal effects of natural killer (NK) cells and lymphocyte activated killer (LAK) cells (Rich et al., 1991, Nicod et al., 1989, Bordignon et al., 1982, Roth and Golub, 1989).

AMs also respond to T cell products. They become activated during acute lung infection (probably due to the influx of T cells into the airways) and exhibit alterations in chemotaxis, cytotoxicity, phagocytosis and secretion of nitrogen/oxygen intermediates, TNFα, IFN and PGE (Holt et al., 1982, Johansson et al., 1997, Chandler et al., 1988, Chandler and Brannen, 1990, Steinmuller et al., 2000, Bilyk et al., 1988, Wizemann and Laskin, 1994, Liu et al., 1997).

The unique function of AMs, relative to macrophages from other sites and tissues, has also been documented using gene expression analysis (Ge et al., 2005, Freeman et al., 2012, Heng and Painter, 2008, Khattra et al., 2007, Gautier et al., 2012, Hume et al., 2010, Mabbott et al., 2010, Kapetanovic et al., 2013). Recent studies compared the expression profiles of AMs with those of blood and marrow-derived cells in the pig (Kapetanovic et al., 2013, Freeman et al., 2012). The AMs were clearly distinct in both basal gene expression and response to bacterial LPS. In particular, AMs constitutively expressed a range of C-type lectin receptors implicated in phagocytosis. Interestingly, the genes that were unique to the AMs were apparently not expressed by intestinal macrophages. These findings further support the theory that AMs have a unique profile compared to other types of tissue macrophages.

1.6 The equine macrophage and the immune system

1.6.1 Equine bone marrow derived and monocyte derived macrophages

In mice, macrophages are routinely generated *in vitro* from bone marrow progenitors for functional studies (Corraliza et al., 1995, Raza et al., 2008). This approach has recently been extended to the pig, expedited by an ability to freeze cells and culture them following recovery (Kapetanovic et al., 2012), but has not yet been applied to horses. Bone marrow colony assays have been described, and used to study the transforming actions of equine infectious anaemia virus (Swardson et al., 1992). Furthermore, a macrophage cell line (e-CAS cells) has been derived from equine

bone marrow cells (Werners et al., 2004) and subsequently shown to have phagocytic capabilities and responsiveness to CSF2 and LPS.

In the horse, blood is more easily accessed than bone marrow. Consequently, there have been several studies on equine monocytes derived from peripheral blood mononuclear cells (PBMCs) and their ability to produce proinflammatory cytokines, such as TNFα and IL1, after LPS stimulation (May et al., 1990, Morris, 1991). Compared to AMs, equine PBMCs appear more sensitive to low concentrations of LPS, potentially consistent with desensitization of AMs by chronic low level stimulation (Grunig et al., 1991). Moreover, another study also revealed transforming actions of equine infectious anaemia virus on equine blood monocytes (Raabe et al., 1998).

In humans and mice, the macrophage response to LPS can be mediated via two different pathways; a myeloid differentiation primary response gene 88 (MyD88) dependent pathway which induces an inflammatory response and/or a MyD88 independent pathway which stimulates the induction of type I *IFN* and *IFN* inducible genes (Kawai et al., 2001, Kawai and Akira, 2007). These pathways appear to be conserved in horses. *TNF*, *IL1β*, *IL6* induction in equine macrophages/monocytes was linked to activation of the MyD88 pathway, while interferon alpha (*IFNα*), interferon gamma-induced protein 10 (*IP10*) and regulated on activation, normal T cell expressed and secreted (*RANTES*, also known as *CCL5*) induction were related to the TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent signaling pathway (Figueiredo et al., 2009, Bjorkbacka et al., 2004, Hirotani et al., 2005). Even high concentrations of LPS did not significantly activate TRIF-dependent gene expression of *INFα*, *IP10*, *RANTES* or *TNF* receptor-associated factor 1 (*TRAF1*) in equine monocytes, suggesting that, in contrast to other studied mammalian-derived cells, the response of equine monocytes to LPS mainly occurs via the MyD88 pathway (Figueiredo et al., 2009).

As demonstrated in other mammalian-derived cells, TLR2 ligands induce a mild inflammatory response by equine PBMCs, compared to the much greater response to TLR4 agonists (Fureix et al., 2012, Gombart, 2009). Equine monocytes are also

responsive to TLR3 stimulation with double stranded RNA [polyinosinic polycytidylic acid (Poly IC)] (Kwon et al., 2010), which is dependent upon the TRIF adaptor. In contrast, horse monocytes do not apparently express TLR5, which explains their lack of response to flagellin (Kwon et al., 2011).

1.6.2 Equine peritoneal macrophages

The peritoneal cavity is another major source of macrophages for functional studies in rodents, in many cases following injection of an irritant or infectious agent (Nomura et al., 2000). It has also been used as a source of human macrophages (Halme, 1989) and pigs (Paul et al., 1979). The function of peritoneal-derived macrophages is unclear; although it is likely to be influenced by their proximity to the gastro-intestinal organs and may act as a defence against any breach of the integrity of the intestinal wall which may result in exposure to microflora and various bacterial-derived products (Qin et al., 2010). Equine peritoneal macrophages stimulated with LPS produced a significant amount of prostanoids and proinflammatory cytokines (Morris and Moore, 1987, Morris et al., 1990, Morris et al., 1992), but did not produce NO (Hawkins et al., 1998). The LPS-induced response could be suppressed by high concentrations of dexamethasone (100 μM), as well as by the anti-inflammatory cytokine IL10 (Morris et al., 1991, Hawkins et al., 1998). Interestingly, LPS-induced TNF production from PMs harvested from healthy horses was significantly increased compared to PMs derived from horses with acute gastrointestinal disease, leading to the suggestion that PMs harvested from endotoxaemic horses are likely to exhibit early endotoxin tolerance (Barton et al., 1996).

1.6.3 Macrophages of the equine lung

Bronchoalveolar lavage in horses is a practical and commonly used method of obtaining macrophages without causing harm to the animal. Consequently, there are several published studies of AMs in this species. Bacteria such as *Streptococcus*

zooepidemicus, *Streptococcus pneumoniae* and *Pasteurella/Actinobacillus species* constitute opportunistic pathogens in the equine airway, triggering the respiratory immune system and inducing inflammation (Wood et al., 1993, Burrell et al., 1996). LPS, a significant component of organic dust derived from equine bedding and forage, is considered a major factor for the induction of airway inflammation in stabled horses (Andonegui et al., 2003).

Werners and Bryant (2012) reviewed the limited literature relating to structure-function relationships amongst pattern recognition receptors in horses (Werners and Bryant, 2012). Early studies found that equine AMs produced large amounts of TNFα in response to LPS, but were significantly less responsive to bacterial LPS than blood monocytes, requiring 100-fold higher concentration to induce procoagulant activity (Grunig et al., 1991). Suri *et al* (2006) demonstrated that TLR4, but not TLR2, is constitutively expressed in healthy horse lungs, with TLR2 being induced by LPS in interstitial macrophages (Suri et al., 2006), a finding also reported in murine, human and porcine macrophages (Schroder et al., 2012, Henneke et al., 2002, Fairbairn et al., 2011). Recently, Waldschmidt *et al* (2013) reported that, in contrast to equine skeletal muscle tissue cells, equine AMs responded efficiently to TLR2, 3, and 4 ligands (Waldschmidt et al., 2013). TLR9, the receptor that recognizes unmethylated CpG oligodeoxynucleotide DNA (Rutz et al., 2004) is expressed by equine lung cells including interstitial and alveolar macrophages and its expression is also upregulated by LPS stimulation (Schneberger et al., 2009). Similarly, TLR9 is expressed in mouse and human lung macrophages and enhanced by LPS (Rutherford et al., 1993, Schneberger et al., 2013).

Lung macrophages may be the primary or secondary site of infection and/or the source of inflammatory cytokines in several important viral diseases including equine arteritis virus, equine influenza, and equine herpesvirus 2 (Schlocker et al., 1995, Moore et al., 2003). Both interstitial and alveolar macrophages are thought to contribute to airway inflammation in recurrent airway obstruction (RAO), a common environmental respiratory disease in adult horses. Certain hay dust-derived components, including endotoxin and fungal spores, activate macrophages and induce chemokines such as IL8 and macrophage inflammatory protein 2 (MIP2) or

CXCL2 (Laan et al., 2006, Aharonson-Raz and Singh) and subsequent neutrophil recruitment (Baggiolini, 1992, Yan et al., 1998, Geiser et al., 1993, Thomsen et al., 1991, Watanabe et al., 1991, Franchini et al., 2000, Franchini et al., 1998). The neutrophil accumulation in turn leads to free radical and protease-mediated tissue damage, a process also seen in human studies (VanWetering et al., 1997, Dallegri et al., 1995, Cavarra et al., 1996).

There is some debate as to whether NO is amongst the free radicals produced by horse macrophages. NO, a product of inducible nitric oxide synthase (iNOS), is a major effector secreted by activated macrophages in rodents, but is not produced by human or porcine macrophages (Kapetanovic et al., 2012, Schneemann et al., 1993). Human and porcine macrophages stimulated with LPS instead metabolise tryptophan through the induction of indoleamine dioxygenase (encoded by the *IDO* gene), kynurenine hydroxylase and kynureninase (KYNU), while mouse macrophages do not use this pathway (Silva et al., 2002, Mellor and Munn, 2004, Kapetanovic et al., 2012). Whilst Hammond *et al* (1999) reported an increase in *iNOS* mRNA in equine AMs after LPS challenge and suggested that the enzyme might be a therapeutic target (Hammond et al., 1999a), others did not reproduce this finding (Johnson et al., 1997).

Whereas normal mouse and human lungs have no PIMs, they are detected in the capillary endothelium of horses and other species such as cattle, pig and sheep and are recognized as a member of the MPS (Aharonson-Raz and Singh, Singh and de la Concha-Bermejillo, 1998, Schneberger et al., 2012). Equine PIMs are considered as proinflammatory cells that play a critical role in equine lung inflammation, since they phagocytose LPS and secrete the proinflammatory cytokines TNFα and IL1β in response to LPS (Parbhakar et al., 2005). Depletion of equine PIMs by gadolinium chloride resulted in a decreased severity of LPS-induced lung inflammation, reflected in a reduced mean pulmonary arterial pressure and lower IL1β production in PIM-depleted horses compared to controls (Parbhakar et al., 2005). Furthermore, depletion of PIMs in horses affected with RAO resulted in a reduction in both clinical symptoms and lung inflammation, characterised by a reduced airway neutrophil count and a decrease in *IL8* and *TLR4* mRNA detected in airway-derived

cells (Aharonson-Raz et al., 2012). Equine PIMs have been shown to express TLR9 and TLR4 and the expression of TLR9 and 2 was augmented after LPS treatment (Schneberger et al., 2013, Suri et al., 2006).PIMs could have a unique role in pulmonary immunity since they are capable of directly responding to inflammatory stimuli within both the airway and intravascular compartments and may partly explain the recognised susceptibility of the horse to endotoxaemia (Schneberger et al., 2012). Although PIMs are not observed in healthy humans, there is evidence of their existence in patients with liver disorders, whereby it has been suggested that they are involved in the hypersensitivity of the airways in response to endotoxaemia (Schneberger et al., 2012).

1.7 Horse as an animal model for human disease research

The mouse is the most widely used animal model for studying human disease, but there are many differences between murine and human pathophysiology (Schneemann et al., 1993, Schneemann and Schoeden, 2007, Junhee et al., 2013). In addition to obvious environmental, anatomical, pathophysiological and genetic differences between mice and humans, there are many distinctions in their innate immune responses (Kapetanovic et al., 2012, Schneemann and Schoeden, 2007, Schneemann et al., 1993, Heinz et al., 2003, Schroder et al., 2012) with specific relevance to mechanistic differences in common inflammatory conditions such as trauma, burns or endotoxaemia (Junhee et al., 2013).Hence, there is an increasing demand for novel animal models (e.g. pig), which more closely resemble human pathophysiology than rodents (Lunney, 2007, Spurlock and Gabler, 2008, Fairbairn et al., 2011), to facilitate research into various human diseases.

Horses have already been used as models for several human diseases, including metabolic syndrome, allergy, chronic obstructive pulmonary disease (COPD), musculoskeletal diseases cancer and even autoimmune uveitis (Turlej et al., 2001, Seltenhammer et al., 2004, Deeg et al., 2007, Hodavance et al., 2007, Koch and Betts, 2007). Although the practical and financial costs associated with equine

research (through feeding, housing and handling) are considerable compared to rodent research and the investigation of large sample populations is problematic, there are significant advantages associated with the use of the horse as a model for human research **(Table 1.5)** (Turlej et al., 2001, Snapper, 1986, Bureau et al., 2000).

Many genes involved in immune function are more closely related between human and horse, than between human and mouse (Milenkovic et al., 2002, Steinbach et al., 2005, Tompkins et al., 2010). Milenkovic and others (2002) identified 113 conserved segments between equine and human genome (Milenkovic et al., 2002). For example, *IL2* showed 72% identity with human and both *IL23* and *IL17* showed greater nucleotide sequence identity with human (89% and 84%, respectively) than mouse (77% and 75%, respectively) (Tompkins et al., 2010). Similarly, equine IFNγ-induced chemokine *CXCL9* has 86% homology with human, but only 74% with the mouse (Hudgens et al., 2011). With specific relevance to macrophages, the equine *CSF1R* is similar to the human *CSF1R*, containing 21 exons which code a 968 amino acid protein within a 30kb region (Roberts et al., 1988, Yarden and Ullrich, 1988, Beck et al., 2005). In addition, Steinbach *et al* (2005) demonstrated both the capacity for equine CSF2 to induce proliferation of a human TF-1 cell line and cross-reactivity of antihuman monoclonal antibodies against CD14, CD163 and CD206 with horse PBMCs (Steinbach et al., 2005).

Chowdhary *et al* (2003) were the first to study the radiation hybrid map of the horse genome and found the level of human-horse synteny (preserved order of genes on chromosomes of related species which results from descent from a common ancestor) to be greater than the level of mouse-horse synteny (Chowdhary et al., 2003). Interestingly, almost half the number of horse chromosomes showed conserved synteny to one human chromosome (Wade et al., 2009). Several other comparative genome studies have shown high conservation between horse and human chromosomes (Raudsepp et al., 1996, Raudsepp et al., 2002, Roenne, 1992). For example, Raudsepp and colleagues (1996) found that the majority of equine chromosomes show homology to human chromosomes, whereas later (2002) the same group demonstrated high conservation of gene order between horse and human chromosome X.

Despite extensive research on equine inflammatory diseases, specific information on the equine MPS is lacking, largely due to the limited availability of appropriate molecular reagents. Consequently, many previous studies have relied on the successful use of cross-reactive human reagents (Steinbach et al., 2005, Ibrahim et al., 2007). With the development of genomic/transcriptomic resources for the horse (http://www.affymetrix.com, http://www.uky.edu/Ag/Horsemap) (see more discussion below) and emerging data from numerous other large mammals (human, pig, cattle, sheep), it is now increasingly possible to address what is known, and what is not known, about the innate immune system of the horse in comparison to other species.

Horses are valuable animals requiring considerable investment from their owners. This is particularly true of racing Thoroughbreds, in which morbidity of any kind can result in substantial financial losses. Horses can suffer from an extensive range of infectious and inflammatory diseases, some of which are zoonotic, others of which share certain clinical and pathological features with equivalent human diseases (Couetil et al., 2007, Sitterle et al., 2012, Traversa et al., 2012). More than 100 equine hereditable conditions may serve as models for human disorders, including inflammation, muscular or fertility disorders, osteoarthritis and even depression (http://omia.angis.org.au, 2009, Carnevale, 2008, Fureix et al., 2012). Furthermore, racehorses resemble elite human athletes and can suffer similar consequences in terms of repetitive injuries and exercise-associated pathology, including arthritis and respiratory tract infections. Therefore in using the horse as an appropriate model for human disease, there is the likelihood that discoveries will have direct benefits for equine health as well.

Comments		*Horse*	*Mouse*	*References*
General	Body, organ, liter size	Closer to human	Less	(Ibrahim et al., 2007, Steinbach et al., 2005)
	Breeding costs	High	Low	
	Sample population, possible experiment repetition	Small	Big	
	Availability of immunological tools/reagents	Limited	High	
Genetic	Homology of protein coded genes with human	High	Low	(Milenkovic et al., 2002, Chowdhary et al., 2003, Raudsepp et al., 1996, Raudsepp et al., 2002)
	Human CD16 is replicated	Replicated	Not replicated	
	Syntenies with human	High	Low	
	Chromosome conservation with human	High	Low	
	Genome annotation	Poor	High	
Macrophage Biology	$CD14^{+}CD16^{+}$ monocytes observed in humans	Detected	Not detected	(Fingerle et al., 1993, Sunderkotter et al., 2004, Noronha et al., 2012, Hawkins et al., 1998, Weinberg et al., 1995, Ito et al., 2005)
	No nitric oxide but indoleamine-pyrrole 2,3-dioxygenase production by human macrophages	Similar	Less similar	
Diseases	>100 hereditary conditions served as models for humans	Yes	No	(Morrison and Ulevitch, 1978, Morris, 1991, http://omia.angis.org.au, 2009, Capomaccio et al., 2011, Langley and Morris, 2009, Koch and Betts, 2007, Fureix et al., 2012, Turlej et al., 2001, Shin et al., 2009, Hodavance et al., 2007)
	Musculoskeletal disorders-osteoarthritis	Common	Less common	
	Human endotoxaemia -sepsis pathophysiology	Similar	Less similar	
	Asthma – Chronic obstructive pulmonary disease pathophysiology	Similar	Less similar	
	Human exercise pathophysiology / immunology	Similar	Less similar	
	Human infectious diseases	Similar	Less similar	
	Human depression	Similar	Less similar	
	Human metabolic diseases	Similar	Less similar	

Table 1.5 : Comparisons of horse and mouse models for human diseases.

1.7.1 Genome and microarray analysis in the horse

The development of useful transcriptomic resources for the horse was dependent upon genomic sequences. Efforts to sequence the equine genome started in 1995 with the Equine Gene Mapping Workshop (Ramery et al., 2009). In 2006, an attempt was made to derive a high quality genome sequence from a female thoroughbred (http://www.board.mit.edu/mammals/horse). Almost 2.7 billion bp of genome sequences were released by the Broad Institute (http://www.broad.mit.edu/mammals/horse). Some parts of the horse genome remained difficult to sequence (Ramery et al., 2009) and the assembly remains a work in progress. Approximately 95% of it has been anchored to chromosomes (autosomes 1-31 and X) with the rest concatenated into a virtual chromosome ''*chrUn*'' separated by gaps of 1,000 bp (http://genome-euro.ucsc.edu/cgi-bin/hgGateway?db=equCab2&redirect=auto&source=genome.ucsc.edu). Gene annotation is currently in progress by the equine genome research community (www.uky.ed/Ag/Horsemap). The need for establishing commercial and reliable genomic technologies such as microarrays or next generation sequencing for equine gene expression experiments, as well as proteomics, led to the improvement of the level of horse annotation, since it is a prerequisite for deriving valuable information from these studies. However, hitherto the field equine genomic annotation remaines challenging (www.affymetrix.com) (Bright et al., 2009).

Earlier studies of the equine transcriptome relied on "home-made" gene expression arrays. A study of synoviocytes was based upon 3000 provisionally annotated genes (Gu and Bertone, 2004). LPS treatment on synoviocytes induced 84 inflammatory and noninflammatory genes (Gu and Bertone, 2004). Based on these results and a few studies that followed, a master genelist was developed and facilitated gene annotation (Santangelo et al., 2007, Colenman S.J et al., 2007, Noschka et al., 2009, Mienaltowski et al., 2008). An early attempt to construct a multi-tissue gene expression atlas followed (Huang et al., 2008b) along with efforts to both correlate gene expression with pathology in equine osteoarthritis [using microarray technology (Smith et al., 2006)] and to identify markers of osteogenic differentiation in horses induced by bone morphogenic proteins 2 and 6 (Zachos et al., 2006). All of these

studies were limited by the extent and annotation of the available expression arrays at the time.

Human and mouse microarrays have been applied in several equine studies (Ing et al., 2004, Michelle R. Mouse et al., 2002, Barrey et al., 2006). Clearly, some probes fail to hybridise but the annotation of probesets on the arrays is superior. Several studies used this approach to provide a rather limited view of nucleated cells derived from peripheral blood (Barrey et al., 2006), airways of horses with RAO (Ramery et al., 2008) and normal or pathologic tendon tissue (Nomura et al., 2007). Murine microarrays have also been employed to study gene expression in healthy equine muscles (Mucher et al., 2006). Both human and mouse microarrays were used on mRNA extracted from equine blood cells in order to shed some light on how exercise affects the physiology and metabolism of the horse (Barrey et al., 2006). Around 12,000 horse-human orthologues have been identified, which enables a comparative approach to assessing the significance of changes seen in association with disease in horses (Glaeser et al., 2009). Capomaccio *et al* (2010) studied the effect of intense exercise on racehorses and investigated the gene expression on PBMCs before, immediately after and 24h following racing using equine/murine oligonucleotide microarray (Capomaccio et al., 2010). This microarray was established based on the combination of the home made equine long oligonucleotide microarray and the open access of human and mouse long oligonucleotide arrays as described in an earlier horse study (Barrey et al., 2009). They found an exercise-associated difference in gene expression, related mainly with inflammatory and immunological mechanisms (Capomaccio et al., 2010). Recently, horse microarrays were also used for investigating the gene profile of equine immature and mature DC subsets, confirming the hypothesis that they consist of two clearly distinct populations (Moyo et al., 2013).

Moreover, short RNA-seq sequences were used to detect extended period of exercise induced genes in equine skeletal muscles (McGivney et al., 2010), while recently, RNA-seq analysis was successfully used to study exercise related gene in equine blood and muscle and interestingly, three exercise involved genes were related with the evolutionary history of Thoroughbreds (Park et al., 2014). RNA-seq has also

been used to study horse development by screening the horse blastocyst (Iqbal et al., 2014) and sperm transcriptome (Das et al., 2013). Other RNA-seq studies have been performed to explore several equine disorders, including congenital stationary night blindness and leopard complex spotting in the horse (Bellone et al., 2013), osteoarthritis (Peffers et al., 2013) and in order to assess the *Corynebacterium pseudotuberculosi* strain transcriptional activity. RNA-seq also represents a valuable tool for improving the annotations of species that have incomplete annotation , such as the horse (Coleman et al., 2013).

A few equine studies have already used proteomics to investigate the pathogenesis of equine diseases, such as arthritis (Williams et al., 2013, Chiaradia et al., 2012), equine recurrent uveitis (Hauck et al., 2012, Degroote et al., 2012, Hauck et al., 2010, Deeg, 2009, Deeg et al., 2007), endometritis (Wolf et al., 2012), RAO (Racine et al., 2011), Potomac horse fever (Gibson et al., 2011), exertional rhabdomyolysis (Bouwman et al., 2010) and *Trypanosoma evansi* infection (Roy et al., 2010). Interestingly, proteomics were also used to investigate the presence of 582 proteins in cell free equine BALF and showed that major homeostatic processes, such as carbohydrate metabolism and cell to cell signalling or function take place in the alveoli (Bright et al., 2011).

1.8 The horse as an elite athlete

The horse is an excellent athlete. Horses evolved living on ancient prairies, where they had to possess sufficient endurance to travel long distances in order to seek water and food whilst maintaining the ability to undertake short episodes of high speed to escape predators (Hinchcliff and Geor, 2004). Over numerous generations, enhancement of their speed was extremely important for their survival (Hinchcliff and Geor, 2004). After their domestication by humans, these attributes (speed and endurance) and other attributes (strength, size), were improved and modified by selective breeding, dependent on the intended purpose of use (Hinchcliff and Geor, 2004). Heavy breeds were bred for strenuous work, like pulling burdens, and lighter ones for transportation and later for racing. The athletic ability of horses is a result of

numerous physiological characteristics; some are independent of training, such as lung size, and others are training dependent, such as blood volume (Hinchcliff and Geor, 2004). Horses also have a greater aerobic capacity compared to other domesticated animals (goat, cows and dogs), a greater ability for intramuscular storage of energy substances (e.g. glycogen), higher intramuscular mitochondrial volume and a great efficiency of thermoregulation. Besides, they have an extensive surface for gas exchange within their large lung, and a high oxygen-carrying capacity in the blood, which can be enhanced during exercise by splenic contraction (Hinchcliff and Geor, 2004). This superior capacity to transport oxygen from inhaled air to muscles is vital in exercise.

1.8.1 Pathophysiological changes during exercise

During exercise, numerous physiological changes occur in the horse **(Table 1.6)**. Muscle contraction requires large amounts of adenosine triphosphate (ATP); thus the metabolic rate is raised to replace spent ATP. ATP synthesis is based on substrate and oxygen supplies, with the substrate mainly derived from carbohydrates in muscle (glycogen) and blood (glucose). These energy supplies are regulated by a variety of hormonal changes that occur during exercise; in particular, a decrease in blood insulin concentration and an increase in catecholamine, cortisol and glucagon concentrations. Hepatic glycogenolysis and gluconeogenesis generates only a small portion (<10%) of the carbohydrates required during intense exercise; the rest is obtained from muscle glycogen stores. Oxygen transportation is also essential during training and is achieved by the coordination of the respiratory and cardiovascular systems. Alveolar ventilation, blood oxygen concentration, splenic contraction and regulation of heart rate constitute the main determinants of oxygen delivery to the tissues, all of which are affected by the intensity of exercise. Muscle contraction also leads to increases in body temperature which, during an acute bout of exercise, may exceed 42^0C, leading to heat shock and morbidity. Furthermore, exercise results in the synthesis of many functional and structural proteins, which have definable effects on the immune system (Kenneth and Raymond, 2008). These will be discussed in the next section **(Section 1.8.2)**.

Physiological changes	During exercise	Recovery Period	References
Heart rate	↑	↓	(Kenneth and Raymond, 2008)
PCV	↑	Recovered 1h post race	(Robson et al., 2003)
Body temperature	↑	↓	(Kenneth and Raymond, 2008)
Metabolism	↑	↓	(Kenneth and Raymond, 2008)
Oxidative capacity	↑	↓	(Kenneth and Raymond, 2008)
Oxygen consumption	↑	↓	(Kenneth and Raymond, 2008)
Blood cortisol	↑	Recovered 1h post race	(Robson et al., 2003, Wong et al., 1992)
	↑	Recovered 11.30h post exercise	(Rossdale et al., 1982)
	↑	Recovered 5 days post exercise	(Huston et al., 1986)
	↑	Recovered 1 day post exercise	(Adamson and Slocombe, 1995)
Blood catecholamines	↑	Recovered 30 min post exercise	(Adamson and Slocombe, 1995)
BALF cortisol	↑	Not available	(Huston et al., 1986)
BALF lipid	↑	Remained high even 5 days post exercise	(Huston et al., 1986)
Blood glucose	↓	Recovered 1h post race	(Robson et al., 2003)
Plasma glutamine	↑	↓ and recovered 24h post race	(Routledge et al., 1999)
	No change	No change	(Robson et al., 2003)
Total blood protein	↑	Recovered 1 day post race	(Robson et al., 2003)
BALF protein	No change	No change	(Huston et al., 1986)
BALF surfactant	↑	Remained high 5 days post exercise	(Huston et al., 1986)

Table 1.6: Physiological changes in response to exercise.

1.8.2 Exercise immunology

Stress is an important factor that may induce numerous pathophysiological changes in the body. Four main stress induced pathways are described to affect the immune

system in athletes: namely, (1) the direct effect of cytokine production, such as IL6, by muscle cells; (2) the activation of the well described hypothalamic-pituitary-adrenal axis (HPA); (3) the extra-adrenal pathways associated with neuropeptides and neurotransmitters; (4) autonomic nervous system [ANS] activation **(Figure 1.6)** (Horohov, 2004, Griffin, 1989).

HPA activation is initiated by the secretion of the corticotropin releasing hormone (CRH) and vasopressin by the brain, resulting in adrenocorticotropic hormone (ACTH), growth hormone (GH) and β endorphin production by the anterior pituitary gland. ACTH subsequently results in cortisol, encephalin and catecholamine release by the adrenal cortex. Increased cortisol release, a chronic response to exercise stress, can cause a decrease in blood lymphocyte numbers and neutrophilia, while increased catecholamine release, an acute response to exercise stress, regulates neutrophil activity in association with growth hormone (Pedersen et al., 1997, Chao et al., 2005). Glucocorticoids also induce gluconeogenesis, a metabolic pathway that produces glucose from non-carbohydrate carbon substrates essential for central nervous system function, as well as for other activities in the body (Chao et al., 2005, Griffin, 1989).

Under stress, there is activation of the sympathetic nervous system, a phenomenon known as the *''fight or flight syndrome''*. Stressors acting via the cerebral cortex of the brain and the hypothalamus result in the activation of the ANS with subsequent catecholamine release, either directly via norepinephrine secretion from sympathetic nerve endings or indirectly via epinephrine secretion from the adrenal gland following HPA axis stimulation (Griffin, 1989, Axelrod and Reisine, 1984).
In turn, this catecholamine release produces vasoconstriction and splenic contraction, leading to splenic-derived erythrocyte and leukocyte release into the circulation (Cross et al., 1988).

Finally, the extra adrenal pathway is less clearly understood (Davies and MacKenzie, 2003). Locally, the release of corticosteroids has both autocrine and paracrine

characteristics and is suggested to result in tissue specific mechanisms (Davies and MacKenzie, 2003).

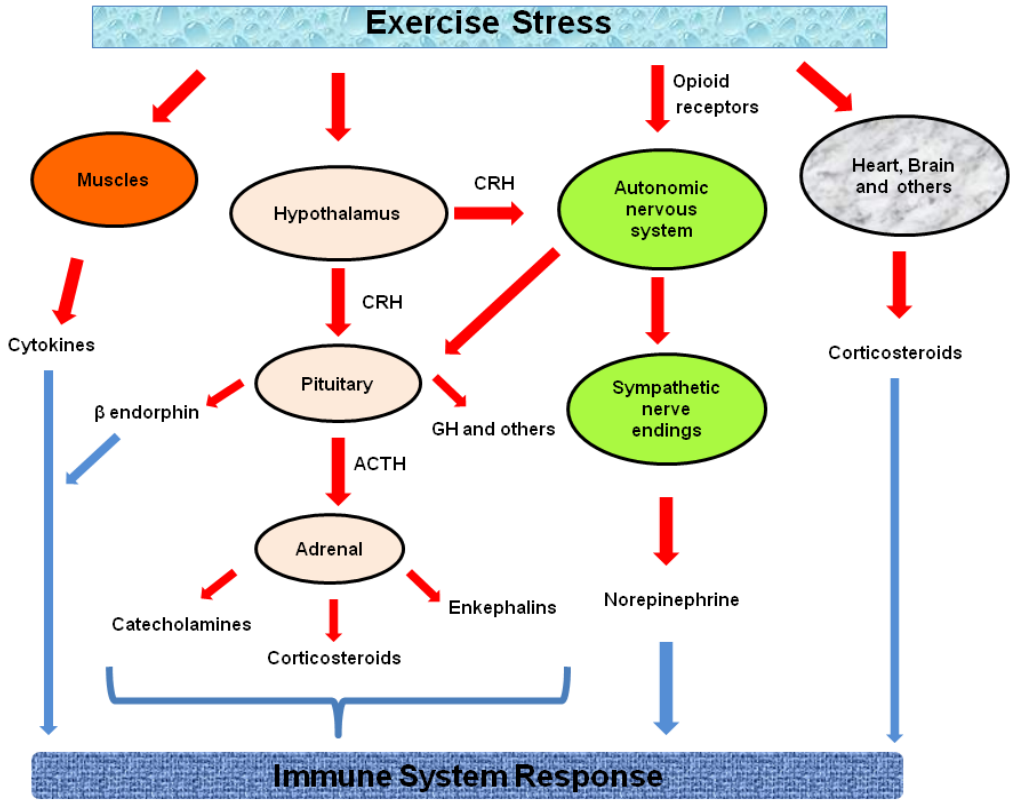


Figure 1.6: The response of the immune system to exercise.

The effect of exercise on the immune system results from the autonomic nervous system and hypothalamic-pituitary-adrenal axis activation, the extra adrenal pathway, as well as from the direct cytokine production by the muscle cells. CRH: corticotropin releasing hormone, GH: growth hormone, ACTH: adrenocorticotropic hormone.

1.8.2.1 Beneficial effect of exercise

Exercise immunology has been a rapidly expanding area of research over the past 40 years, with its subsequent development as a specific discipline with its own international society and dedicated journal (http://www.isei.dk/, Walsh et al., 2011). Moderate and regular physical activity clearly has beneficial effects on humans. For example, a positive effect of exercise on various chronic inflammatory conditions such as cardiac and respiratory disorders, type II diabetes, atherosclerosis and several

types of cancer is consistently reported (Woods et al., 2009, Blair et al., 2001, Troosters et al., 2005). Furthermore, a better prognosis and a lower risk of coronary heart disease, stroke, cardiovascular disease and colon cancer are reported in more physically active individuals (Blair et al., 2001). Numerous studies have detected a relationship between inactivity in both young and elderly individuals and intermittent claudication associated with a low level of systemic inflammation (Abramson and Vaccarino, 2002, Geffken et al., 2001, Tisi et al., 1997). In elderly individuals this phenomenon has been termed inflammaging (Franceschi et al., 2000) and physical activity has been shown to affect this low grade inflammation (Tisi et al., 1997, Geffken et al., 2001, Abramson and Vaccarino, 2002). Even though similar studies have not been performed on horses, a beneficial effect of exercise on equine athletes is likely to occur.

The inflammatory effect induced by moderate exercise may involve IL6, a cytokine with both proinflammatory and anti-inflammatory properties (Tilg et al., 1997, Starkie et al., 2003, Gabay, 2006, Petersen and Pedersen, 2005). IL6 is highly expressed by contracting muscle cells during exercise and its expression is further enhanced by low muscle glycogen levels; thus this cytokine is considered a potential regulator of hepatic glucose metabolism (Keller et al., 2001, Steensberg et al., 2001a). IL6 controls lipid metabolism, induces lipolysis and fat oxidation independent of circulating hormonal changes and without causing hypertriacylglyceridemia (Petersen et al., 2005, van Hall et al., 2003). Furthermore, exercise-associated IL6 secretion by skeletal muscle cells inhibits the production of TNFα, a cytokine involved in a number of pathogenic conditions such as insulin resistance and atherosclerosis (Blair and Brodney, 1999, Starkie et al., 2003). Even after exposure to endotoxin, TNFα release in human subjects was inhibited by either physical exercise or rhIL6 infusion at physiological concentrations (Starkie et al., 2003). IL6 can also trigger cortisol release and the production of anti-inflammatory cytokines (e.g. IL1RA and IL10) and delay C reactive protein secretion (Steensberg et al., 2003). The low lymphocyte counts detected during the recovery period following exercise may partly be attributed to elevated plasma cortisol levels, induced by IL6 (Steensberg et al., 2001b, Steensberg et al., 2003). IL6 has also been

shown to induce the production of soluble TNF receptor (sTNFR), a principal inhibitor of TNFα activity (Tilg et al., 1994) **(Figure 1.7)**.

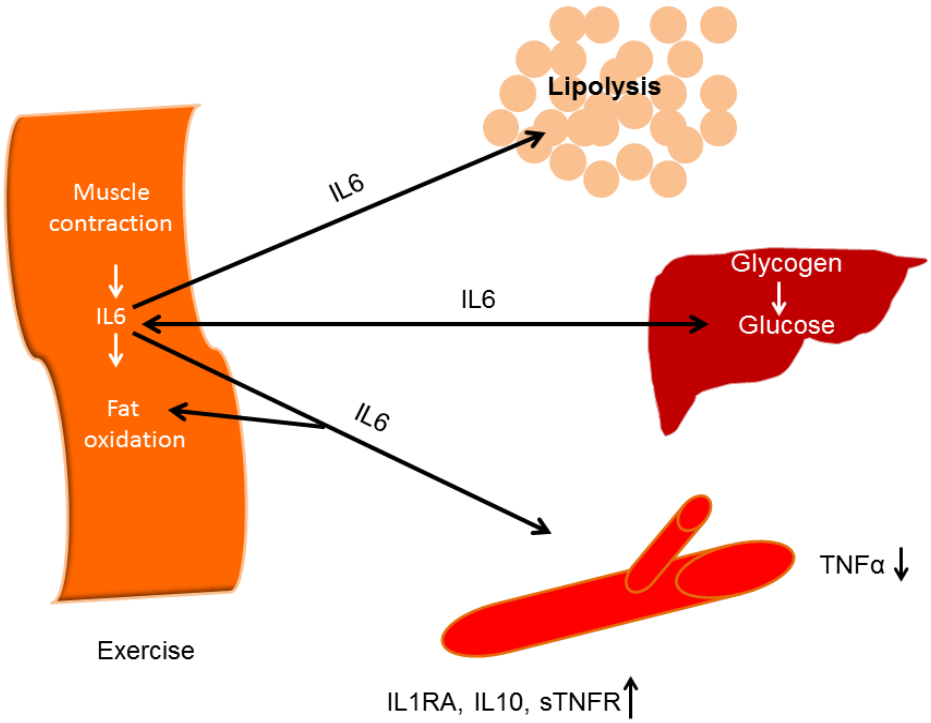


Figure 1.7: IL6 activities during exercise.

During physical activity IL6 is produced by skeletal muscle cells in high levels. IL6 plays an important role in glucose homeostasis and is an important modulator of fat metabolism as it induces lipolysis and fat oxidation. IL6 triggers the production of the anti-inflammatory cytokines IL1RA, IL10, sTNFR and inhibits the production of TNFα. Figure adapted from Marie et al. 2005.

Epinephrine also contributes slightly to IL6 production. However the suppression of endotoxin-induced TNFα release observed in humans pre-exposed to epinephrine (vanderPoll et al., 1996) is considered to be independent of IL6 (Steensberg et al., 2001b, Petersen and Pedersen, 2005). In a comparative study of humans and horses, the level of *IL6* mRNA expression by PBMCs was found to be greater in both species during training (Capomaccio et al., 2011, Colahan et al., 2002),while *IL6R* was more so in horses, which may thereby have superior means of responding to exercise (Capomaccio et al., 2011).

Antioxidants are another potential anti-inflammatory mechanism induced by exercise (Leeuwenburgh and Heinecke, 2001, Abramson and Vaccarino, 2002). Although exercise can result in oxidative stress, long term training results in antioxidant production thus preventing any oxidant-induced chronic tissue damage (Leeuwenburgh and Heinecke, 2001, Leeuwenburgh et al., 1999, Powers et al., 1994, Robertson et al., 1991). The benefits of exercise may also partly relate to its effect on lung surfactant (van Iwaarden et al., 1990, Huston et al., 1986, Rooney, 1985). In horses, BALF surfactant levels increase significantly following training, remaining elevated even after five days (Huston et al., 1986).

1.8.2.2 Deleterious effect of exercise

Where moderate exercise improves immunity in humans, especially in the elderly, long-term strenuous exercise has the opposite effect (Bruunsgaard and Pedersen, 2000, Field et al., 1991, Fitzgerald, 1991). The same is apparently true in horses. Histopathological lesions have been observed in the lungs of racehorses, reflective of conditions such as exercise-induced pulmonary haemorrhage (EIPH), and pulmonary inflammation (McKane and Slocombe, 2002, Collins et al., 1994). The phenomenon of overtraining has been widely studied in humans. There are several inconsistencies in the terminology used and accurate diagnostic markers for this syndrome remain to be identified (Purvis et al., 2010, Meeusen et al., 2013). The diagnosis is mainly based on the athlete's psychological profile (McKenzie, 1999) although clinical symptoms might reflect sympathetic or parasympathetic nervous system alterations, including severe loss of appetite, fatigue, performance decrements, insomnia, excitability, anxiousness, weight loss, muscle and joint pain, hypoglycemia in non-diabetic athletes, lack of concentration and enthusiasm for the sport, apathy and depression (Purvis et al., 2010, Meeusen et al., 2013). Since psychological status and mood cannot easily be estimated in horses, the diagnosis is based on other measured parameters such as performance decrements, weight loss and reduced oxygen consumption (Hamlin et al., 2002). Recently, new analytical technologies, such as microarrays and proteomics, have been proposed as potential tools for the detection of subclinical performance or even a means by which the training status of the horse

can be monitored (te Pas et al., 2013, Scoppetta et al., 2012). Unfortunately, no reliable biomarkers have been identified to date.

Exercise-induced immunosuppression has been studied in humans (Cox et al., 2007, Abbasi et al., 2013, Cox et al., 2008) and laboratory animals (Davis et al., 1997, Leeuwenburgh et al., 1999, Woods et al., 1997) and to a lesser extent in horses (Raidal et al., 2000, Wong et al., 1990) and dogs (McKenzie et al., 2007, Clark et al., 1997). Much of the data applied to the field of human exercise immunology have been derived from a number of animal studies (Kakanis et al., 2010, Keast et al., 1988, Raidal et al., 2000). Such studies have helped to identify a variety of immunological derangements associated with both acute and chronic exercise, including exercise-associated effects on cell migration and margination, mucosal IgA secretion, cellular function and cell surface receptor expression (Walsh et al., 2011). The recognition of such alterations has in part helped to reveal the mechanisms which may lead to an exercise-associated increased susceptibility to opportunistic infection, commonly referred to as the *"open window"* theory **(Figure 1.8)** (Walsh et al., 2011).

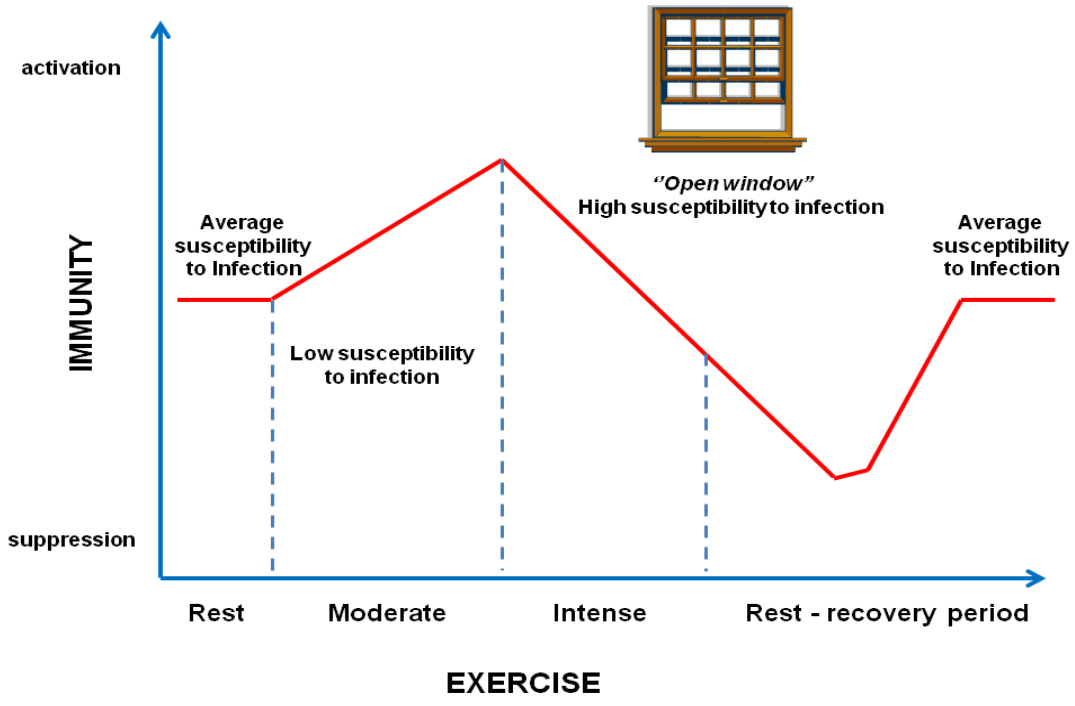


Figure 1.8: The immune response to exercise and the "open window" theory.

Intense exercise can increase the risk of upper respiratory tract infections in humans and augment latent infections (MacKinnon, 2000, Nieman, 1994). Humans and horses may differ with respect to the impact of exercise on respiratory tract infections and the results obtained in published studies in both species are often contradictory (Cox et al., 2008). This can partly be attributed to differences between studies with respect to the training protocols applied, the sampling time points (e.g. during or after active exercise or during a prolonged "training period"), the types of samples collected and the experimental methodologies used (Robson et al., 2003, Huston et al., 1986). Such differences in study design can significantly impact on the value of inter-study data comparisons due to the numerous physiological changes, mainly neuroendocrine and metabolic, that occur during physical activity (Jonsdottir, 2000, Venkatraman and Pendergast, 2002).

1.8.2.3 The effect of exercise on leukocyte distribution and function

During exercise, muscle contraction results in hyperthermia and sympathoadrenal activation, which leads to immune cell redistribution (Rhind et al., 1999). Therefore, most equine studies have focussed on the distribution of blood cellular components (monocytes, neutrophils, lymphocytes) (Rossdale et al., 1982, Jensen-Waern et al., 1999, Robson et al., 2003) and those looking at the response of the innate immune system using *in vitro* assays to measure phagocytic or oxidative burst activity of neutrophils/monocytes (Horohov, 2004, Hines et al., 1996). Limited studies on the effect of exercise on the adaptive immune response have investigated lymphocyte proliferation and function (Horohov et al., 1999, Hines et al., 1996). The effect of exercise on the innate immune system, first appearing at the beginning of the workout, seems to be biphasic in both humans and horses (Kakanis et al., 2010, Rossdale et al., 1982). Short exercise-induced changes in the immune system include a transient increase in both neutrophil and lymphocyte numbers (Rhind et al., 1999, Robson et al., 2003, Kakanis et al., 2010) followed, in both humans and horses, by a decrease in circulating lymphocytes below normal levels approximately one to two hours after exercise, a phenomenon attributed to high plasma cortisol levels (Nesse et al., 2002, Pedersen et al., 1997). Results of various studies are summarised in **Table**

1.7. The level of leukocytosis depends on the magnitude of the physical activity, with more intense exercise resulting in a greater increase in leukocyte numbers post exercise (Brenner et al., 1998).

	Post exercise	Recovery Period	References
Lymphocytes	↑	↓ Recovered 1h post race	(Rossdale et al., 1982)
	Not available	↓ did not recover 16h post exercise	(Nesse et al., 2002)
	↓	↑ recovered 1 day post race	(Robson et al., 2003)
$CD4^+$/$CD8^+$ T cell	↓	Not available	(Hines et al., 1996)
Monocytes	↑	Recovered 1h post race	(Robson et al., 2003)
	No change	No change	(Jensen-Waern et al., 1999)
Neutrophils	↑	Recovered 3 days post race	(Robson et al., 2003)
	↑	Recovered 8 days post race	(Jensen-Waern et al., 1999)

Table 1.7: The effect of exercise on leukocyte distribution in horse blood.

In addition to exercise induced blood cell redistribution, changes have also been reported in BALF cell counts. The exercise-associated increase in total BALF cell count has been attributed to increases in both neutrophils and eosinophils, potentially in response to the release of the proinflammatory platelet-activating factor in the airway (McKane et al., 1993, Michelotto et al., 2010). In comparison, other studies have reported either a reduction in the number of total BALF cells after 10 weeks of training (Clark et al., 1995) and three days following exercise (Huston et al., 1986) or no changes in cell number (Wong et al., 1990, Ainsworth et al., 2003a, Raidal et al., 2000).

Leukocyte function may also change in response to exercise (Raidal et al., 2001, Robson et al., 2003). Overall, initial activation of immune cells both during and shortly after training is followed by general suppression of the immune system in

several human and horse studies (Kakanis et al., 2010, Cox et al., 2008, Cox et al., 2007, Gleeson et al., 1999, Robson et al., 2003). In particular, lower immune cell numbers (lymphocytes) and impaired cellular function (phagocytosis, oxidative burst activity) develop in the recovery period following an acute bout of endurance exercise (Kakanis et al., 2010, Robson et al., 2003, Nesse et al., 2002). There have been multiple reports focusing on suppressed function in macrophages in response to exercise, which is linked potentially to lower respiratory tract infections in the horse (Wong et al., 1990, Folsom et al., 2001). The current literature on equine immune cells with emphasis on macrophages/monocytes is summarised in **Table 1.8**.

Strenuous exercise resulted in impaired phagocytosis of equine blood monocytes, an effect which persisted even three days following exercise (Wong et al., 1990). A suppression of AM phagocytic activity during exercise is also documented by several studies (Huston et al., 1986, Wong et al., 1990, Raidal et al., 2000). However, a large degree of inconsistency exists in the literature with respect to reported results (Raidal et al., 2001, Adamson and Slocombe, 1995), a phenomenon which may be explained by the common occurrence of EIPH (McKane et al., 1993). In EIPH, the RBCs tend to form rosettes around AMs and impair phagocytosis, potentially by blocking cell receptors on the AM membranes (Commins et al., 1990). Furthermore, RBC internalization by both alveolar and peritoneal macrophages neutralizes reactive oxygen metabolites, thus potentially impairing their oxidative burst activity (Adamson and Slocombe, 1995, Hand and Kingthompson, 1983).

There are many other variables in these studies which may also partly explain the inconsistent results, including the duration, nature and season of training, the nature of inhaled particles, the age of the horses and the inter-individual variation in macrophage function. It is therefore likely that vigorous exercise can be either pro- or anti-inflammatory in the lung, depending on a variety of circumstances. These results further support the theory that intense exercise may have a negative impact on the pulmonary system of the horse, either directly or via immunosuppression, with a subsequent increase in susceptibility to opportunistic infections.

Activity	Cell type	Finding	References
Viability	Blood leukocytes	No change post strenuous exercise	(Adamson and Slocombe, 1995)
	BALF cells	↓ after the training period, but no change was observed post exercise	(Raidal et al., 2000)
	Alveolar macrophages	↓ till 5 days post exercise	(Adamson and Slocombe, 1995, Huston et al., 1986)
Morphology	Blood granulocytes	No change post strenuous exercise	(Adamson and Slocombe, 1995)
	Alveolar macrophages	No change post strenuous exercise	(Adamson and Slocombe, 1995, Huston et al., 1986)
LAK cell activity	LAK cells	↑ 20min after intense exercise but returned to resting levels 2h post exercise	(Keadle et al., 1993)
Oxidative burst activity	Blood monocytes, neutrophils	↓ and did not captured even 3 days post exercise	(Robson et al., 2003)
	Blood neutrophils, lymphocytes	↑ and during prolonged training decreased	(Raidal et al., 2001)
	BALF granulocytes	↓ 30min post strenuous exercise	(Adamson and Slocombe, 1995)
	BALF derived lymphocytes	↑ post strenuous exercise	(Raidal et al., 2000)
	BALF derived lymphocytes	No change	(Raidal et al., 2001)
	Alveolar macrophages	↑ 30min post strenuous exercise	(Raidal et al., 2000)
	Alveolar macrophages	Varied during the study	(Raidal et al., 2001)
	Alveolar macrophages	↓ at race trained horses compared to those at rest	(Michelotto et al., 2010)
	Alveolar macrophages	No change 30min, 1 or 5 days after strenuous exercise	(Adamson and Slocombe, 1995)

Activity *(Continued)*	Cell type	Finding	References
Phagocytic activity	Blood monocytes, neutrophils	↓ and did not captured even 3 days post exercise	(Wong et al., 1992)
	Alveolar macrophage	↓ and recovered at day 3 post exercise	(Huston et al., 1986)
	Alveolar macrophage	↓	(Wong et al., 1990)
	Alveolar macrophage	↓ 30min post exercise of the training period	(Raidal et al., 2000)
	Alveolar macrophages	No change	(Raidal et al., 2001)
	Alveolar macrophages	↓ at race trained horses compared to those at rest	(Michelotto et al., 2010)
	Blood neutrophils	↓ after 4-7 weeks of intense training	(Buschmann and Baumann, 1991)
	Blood neutrophils	↓ even one day post race	(Jensen-Waern et al., 1999)
	Blood neutrophils	↑ at intense exercise and decreased at overtraining	(Raidal et al., 2001)

Table 1.8: The effect of exercise on equine immune cells, with emphasis on the monocyte / macrophage.

LAK = lymphokine activated killer.

1.8.2.4 Cytokines and other substances in the response to exercise

As noted in the previous section, the short term effect of exercise on leukocyte distribution may resemble an LPS challenge (Rhind et al., 1999). There are several studies focusing on the cytokine expression of immune cells during and after exercise in man, horses and other species (Moldoveanu et al., 2000, Liburt et al., 2010, Keller et al., 2004, Wakshlag et al., 2010). In addition to IL6 (discussed above), IL1, IL8, IL1RA, TGFB1 and TNFα have been detected in human plasma following an acute bout of endurance exercise (Moldoveanu et al., 2000, Abbasi et al., 2013). Similarly, in horses, several proinflammatory (TNFα, IL1β, IL6, IL6R, IL8, IL12) and anti-inflammatory (IL6, IL10, IL1RA) cytokines and the acute phase protein C-reactive protein, were found to be released in high levels following exercise (Horohov, 2004, Liburt et al., 2010, Barton et al., 2003, Capomaccio et al., 2011, Cappelli et al., 2007). The response of PBMCs to racing has been analysed using gene expression arrays (Capomaccio et al., 2010). A list of 132 genes was

induced transiently, but declined to basal levels after 24h (Capomaccio et al., 2010). The induced genes included both proinflammatory and anti-inflammatory genes, such as *TNFα*, *IL18*, *IL8*, *IL1β*, *SOCS3* and *CCL3* (Capomaccio et al., 2010). The same group used next generation sequencing and reported similar results, but identified a number of novel candidate regulators (Capomaccio et al., 2013). Recently, another study examined the effect of training, as opposed to acute exertion. In particular *TLR3* mRNA expression was downregulated in both AM and monocytes and did not normalise during the recovery period (Frellstedt et al., 2014). Moreover, viral challenge of AMs resulted in low levels of *TNFα* and *IFNβ* expression, further supporting the existence of an exercise-associated increased susceptibility to viral infections (Frellstedt et al., 2014).

The high levels of circulating endotoxin observed in both athletes and racehorses after strenuous exercise might explain the release of TNFα by immune cells (macrophages/monocytes) post exercise (Barton et al., 2003, Jeukendrup et al., 2000). Because of sympathetic stimulation during intense exercise, blood flow is diverted from the gastrointestinal tract, potentially resulting in intestinal hypoperfusion and a compromised integrity of the mucosal barrier. Furthermore, increases in body temperature and changes in vessel permeability may also contribute to an increase in endotoxin levels in the circulation (Barton et al., 2003). Moreover, the cytokine release observed in the circulation following strenuous exercise might partly reflect cytokine secretion in tissues resulting from exercise-associated tissue damage (Smith et al., 1992, Woods and Davis, 1994).

1.9 Inflammatory Airway Disease

Inflammatory airway disease (IAD) is a nonspecific inflammatory condition of the equine airways, which is recognized as the most common respiratory disorder in young racehorses in the UK (Wilsher et al., 2006, Wood et al., 2005b). Its prevalence in racehorses has been estimated between 11.2 and 50% (Wood et al., 2005b, Wood et al., 2005a, Burrell et al., 1996). Although young racehorses (2-4 years old) are more prone to develop the disease, under the current diagnostic definition, it can also

present in nonracehorses of any age (Chapman et al., 2000, Wood et al., 1993, Robinson et al., 2006).

The most common clinical signs of IAD are chronic and intermittent coughing and poor racing performance (Chapman et al., 2000). The presence of mucopurulent tracheal secretions with an elevated neutrophil concentration is considered to be the hallmark of IAD (Moore et al., 1995, Sweeney et al., 1992). Pulmonary dysfunction due to lower airway inflammation may also occur (Robinson, 2003) and airway hyper-responsiveness and impaired blood gas exchange are also present according to the disease phenotype proposed by the American College of Veterinary Internal Medicine consensus panel members in October 2003 (Robinson, 2003).

Due to the relatively nonspecific nature of the clinical signs of IAD, diagnosis can often be difficult and is based primarily on cytologic examination of BALF, mainly characterized by either a mild neutrophilia, eosinophilia or mastocytosis or a combination of these (Couetil et al., 2001, Hoffman, 2008). Recently, IAD cases have been subdivided into three main subtypes, depending on their BALF cytological profiles; namely, neutrophilic, mast cell or both (Beekman et al., 2012). Abnormal BALF cytology results have been related to poor performance in all horse types (Fogarty and Buckley, 1991, Moore et al., 1995, Richard et al., 2010b). The BALF differential cell count (DCC) is sometimes used to discriminate between diseases of the horse lung. For example the cytological profile of horses affected with RAO is characterised by a substantial increase in neutrophil ratio in the BALF, usually much higher than in horses with IAD (McGorum et al., 1993, Robinson, 2003). Ultimately, the distinction between these clinically related conditions can only be achieved by other diagnostic means, including lung function tests, environmental manipulation and careful consideration of the history (Couetil et al., 2001). In contrast to RAO cases, no increased respiratory effort is observed in horses affected with IAD at rest (Robinson, 2003), and such an increased effort cannot be induced by exposure to mouldy hay (Dixon et al., 1995).

A characteristic feature of IAD is also a subtle decline in pulmonary function with resultant gas exchange abnormalities during exercise (Sanchez et al., 2005). The

results of standard lung mechanics testing are normal in IAD (Ocallaghan et al., 1987), although impulse oscillometry, a noninvasive method of assessing pulmonary mechanics, can be useful for the early detection of subclinical cases of airway inflammation (Richard et al., 2009). Unfortunately, more sensitive pulmonary function tests are not widely available to clinicians (Couetil et al., 2001). Finally, airway hyper-responsiveness, bronchoconstriction and cough are linked with abnormal BALF profiles in horses with IAD (Hare and Viel, 1998, Hoffman et al., 1998). Interestingly, increased serum concentration of SPD was found in horses affected with IAD (Richard et al., 2012), as well as with experimental bacterial airway infection (Hobo et al., 2007), suggesting that SPD could be used as a serum biomarker for lower airway inflammation in the horse.

Although the exact aetiopathogenesis of IAD is not completely elucidated, a variety of infectious and noninfectious causes have been proposed and reported (Pirrone et al., 2007, Wood et al., 2005a, Holcombe et al., 2001). Training at a young age is an important risk factor for IAD in racehorses (Malakides M and Hodgston, 2003) and stress from transportation, interaction with other horses and housing in poorly ventilated places may contribute to its development (Wilsher et al., 2006, Cardwell, 2009). A strong correlation exists between IAD and high levels of airborne endotoxins from dust sources, poor ventilation and certain meteorological conditions, such as high evaporation level, humidity, high temperatures and wind (Malakides M and Hodgston, 2003). These factors likely increase the load of inhaled proinflammatory agents in the lower airways. Furthermore, increased ventilation during intense exercise also will result in a greater likelihood of inhaling pharyngeal and environmental pathogens or airborne foreign substances (Gordon and Read, 2002, Kakanis et al., 2010).

The incidence of infection with *Streptococcus pneumoniae* and the prevalence of IAD decreases with age (Wood et al., 2005a). Virus infections do not seem to be highly associated with the pathogenesis of IAD (Wood et al., 2005a, Burrell et al., 1996), although the number of viruses tested was not extensive. However, IAD could result from viral-induced immunosuppression, subsequently followed by opportunistic bacterial infections (Burrell et al., 1996, Hannant et al., 1991).

A few studies have been performed to investigate whether a polarised T cell response is involved in the aetiopathogenesis of the disease **(Table 1.9)**. Horses with IAD have an increased expression of proinflammatory cytokine mRNA (*TNFα*, *IL1β* and *IL23*) within BALF derived cells, reflecting an innate immune response, likely to inhaled antigens. In keeping with this interpretation, there was no clear evidence of a polarised T cell response (Hughes et al., 2011). By contrast, another group found both *IL4* and *IFNγ* mRNA in BALF cells (Lavoie et al., 2011), results which were suggestive of both a Th1 and Th2 response in the cases investigated (Lavoie et al., 2011, Lasky and Brody, 1997). Similar findings have also been reported in relation to RAO, an asthma-like obstructive airway disease in horses, suggesting that a common pathologic mechanism may exist between these two diseases (Ainsworth et al., 2003b, Giguère et al., 2002, Lavoie et al., 2011).

By contrast, another group indicated a more Th2-type response of BALF cells derived from horses affected with IAD (Beekman et al., 2012). However, different IAD subtypes may be associated with different immunological mechanisms (Beekman et al., 2012). For example, BALF eosinophilia, common in horses with IAD and mastocytosis, is associated with airway hyperactivity, whereas BALF neutrophilia is associated more with coughing (Hoffman et al., 1998, Bedenice et al., 2008). Interestingly, *IL4* was detected only in the mast cell IAD group suggesting that Th2 cytokines and IgE may participate in this disease subtype (Beekman et al., 2012), whereas the neutrophil chemoattractant *IL17* was detected only in the neutrophilic IAD. Hence, there seems to be a correlation between cytokine production by BALF cells and BALF cytologic profile and, even though both Th1 and Th1 response may be implicated in IAD, subtypes of the disease may implicate different mechanisms.

IAD type	Finding	References
IAD and exercise intolerance	*TNFα*, *IL1β*, *IL4* and *IFNγ*	(Lavoie et al., 2011)
IAD	*TNFα*, *IL1β* and *IL23*	(Hughes et al., 2011)
	IL1β, *IL5*, *IL6*, *IL8* and *IL10*	(Beekman et al., 2012)
Neutrophilic IAD	*IL1β*, *IL5*, *IL6*, *IL8* and *IL17*. *IL1β*, *IL5* and *IL8* were higher compared to the mast cell IAD	(Beekman et al., 2012)
Mast cell IAD	*IL1β*, *IL4*, *IL5*, *IL6*, *IL8* and low *IL12p35*	(Beekman et al., 2012)

Table 1.9: Summary of cytokine mRNA expression by BALF cells derived from horses with IAD.

At present, therapeutic options for the treatment of IAD are limited. These are based primarily on environmental modifications, and anti-inflammatory and antibiotic medication (Bertone, 1997). The aim of the environmental modifications is to reduce exposure of the respiratory system to various potentially proinflammatory airborne antigens. Improving the ventilation system of the stable and using feed and bedding that produce low levels of airborne dust is considered extremely important (Woods et al., 1993, Pirie et al., 2002).

The use of inhaled corticosteroids (e.g. fluticasone or beclomethasone) is a frequent anti-inflammatory therapeutic approach (Rush et al., 2000, Couetil et al., 2005). Although systemic corticosteroid therapy (e.g. dexamethasone or prednisolone) is also commonly used, the risks of undesired side effects are greater compared with the inhalation route of administration (Rush et al., 1998). Additional therapeutic

approaches have included inhaled sodium cromoglycate, a mast cell stabilizer which improves clinical appearance and decreases airway hyper-responsiveness (Hare et al., 1994) and oral IFNα, which has been shown experimentally to reduce respiratory inflammation in Standardbred racehorses with IAD (Moore et al., 1997). Due to the subclinical form of the disease at rest, re-examination of the patient is needed before training recommences (Couetil et al., 2007).

1.10 Preventive measures for exercise induced dysregulation of the immune system

1.10.1 Dietary measures

Glutamine, an amino acid mainly produced in muscle cells and essential for immune cell function and preservation, is decreased in the plasma of both marathon runners following racing or during overtraining (Parry-Billings et al., 1992) and in racehorses following exercise (Routledge et al., 1999). Glutamine oral administration in human athletes resulted in lower risk for infection compared to the control group, suggesting that glutamine dietary supplements are beneficial for the immune system during exercise (Castell et al., 1996). Another nutritional supplement that is suggested to enhance the immune activity in horses is ascorbic acid (Vitamin C) (Snow et al., 1987). Short term supplementation of horses with Vitamin C after long-lasting stress, is beneficial to immune function, although long term supplementation is not recommended (Ralston and Stives, 2012).

1.10.2 Macrophage regulation

Many infectious or autoimmune diseases encountered in veterinary and human medicine are associated with an excessive inflammatory response of immune cells and several methods have been employed to therapeutically or prophylactically suppress or inhibit such responses, particularly targeting macrophages (Nathan, 2002, Ainsworth et al., 2003b). For instance, TNF converting enzyme (TACE) inhibition showed a significant reduction in proinflammatory cytokine (TNF, IL1β

and IL6) production by both equine and human macrophages *in vitro* (Wijnker et al., 2004). Parbhakar *et al* (2005) also used gadolium chloride and successfully depleted equine pulmonary intravascular macrophages and showed a decrease in the severity of LPS-induced lung inflammation (Parbhakar et al., 2005). Additionally, IL4, IL13 and IL10 are shown to be critical for maintaining the balance between Th1 and Th2 immune responses to several infectious pathogens (Barnes, 1998, Bogdan and Nathan, 1993). Jackson *et al* (2004) showed that horse AMs cultured in recombinant equine IL4 produce less mRNA of the proinflammatory cytokines IL8 and IL12 p40 after LPS stimulation than LPS treated cells, indicating that IL4 can modulate AM in response to LPS (Jackson et al., 2004). Conversely, the enhancement of macrophage function could produce therapeutic and/or prophylactic clinical benefits (Tzianabos, 2000). For instance the use of polysaccharides, poly-(1-6)-P-glucotriosyl-(1-3)-P-glucopyranose -glucan and *Bacteroides fragilis* polysaccharide A in a rat model with experimental intra-abdominal sepsis resulted in reduced mortality and abscess formation (Tzianabos et al., 1998).

Many studies have investigated the regulatory mechanisms of macrophage polarisation. Recently, it was shown that M2 polarization was induced in Bruton's tyrosine kinase (Btk) deficient mice after treatment with LPS, an M1 stimulus (Ní Gabhann et al., 2014), since Btk activates NFκB via TLR4 (Horwood et al., 2006). Many earlier studies investigated the regulation of macrophage activation, including the effect of several molecules, such as members of the SOCS family, members of interferon regulated factors (IRFs), members of the signal transducers and activators of transcription (STAT) family and several signaling pathways (Spence et al., 2013, Sica and Mantovani, 2012, Zhou et al., 2014). For example, SOCS2 induces M2 polarisation, SOCS3 the classical phenotype (Spence et al., 2013) and STAT3 and STAT6 the alternative macrophage activation pathway (Sica and Mantovani, 2012). Thus, modulation of macrophage function may provide new insights in order to facilitate treatment measures for infectious and non-infectious respiratory diseases in horses, in particular for infections resulting from antibiotic resistant organisms, autoimmune or nonspecific inflammatory diseases.

One approach to alleviating the effect of exercise would be to adopt the use of immunomodulators (Ziebell et al., 1997, Klier et al., 2011). For example, parapoxvirus ovis (PPOV) is a virus belonging in the Poxviridae family which infects humans, goats and sheep, presenting mainly with acute skin lesions (Haig and Mercer, 1998). Its inactivated form (iPPOV) has been shown to induce both immunostimulatory and immunosuppressive actions (Fachinger et al., 2000). Its immunostimulatory properties include the promotion of antigen presentation, phagocytosis and inflammatory related cytokine production (TNFα, IL2, IL6 IFNγ), whereas its immunosuppressive properties include the induction of anti-inflammatory cytokine production, such as IL4 and L10 (Fachinger et al., 2000, Friebe et al., 2004, Knolle et al., 1998). Thus, iPPOV has been proposed as an efficient antiviral drug, potentially offering new perspectives in antiviral therapeutic options. Its effects on cells derived from several species share many similarities and several studies on its immunomodulating properties have been reported in the literature (Ziebell et al., 1997, Fachinger et al., 2000, Friebe et al., 2004). PPOV acts as a superantigen on porcine PBMCs and was found to induce a MHC II dependent T cell proliferation (Fachinger et al., 2000). In a clinical trial on horses, iPPOV efficiently reduced the severity of respiratory clinical signs resulting from stress and viral and bacterial infections (Ziebell et al., 1997). It is currently marketed under the tradename Zylexis® by Zoetis.

Cytosine phosphate guanine oligodeoxynucleotides (CpG-ODNs) are another immunostimulant widely studied in mice which acts by suppressing the Th2 response and enhancing a Th1 shift, thus providing defence against asthmatic inflammation (Kline, 2007). In horses, a similar immunostimulatory effect of CpG-ODNs on BALF cells was reported and characterised by both IL10 and IFNγ release after CpG-ODN administration (Klier et al., 2011). A subsequent *in vivo* study performed by the same group demonstrated that inhalation of biodegradable, biocompatible and nontoxic gelatin nanoparticle bound CpG-ODN 2216 resulted in enhanced IL10 release by BALF cells (Klier et al., 2012). The reported IL10 release may relate to T reg cell activation, a process shown to inhibit neutrophil infiltration (Montagnoli et al., 2006). Moreover, the stimulation of IL10 and IFNγ release by T reg cells has also

been shown to be protective against the development of airway hyperactivity (Stock et al., 2004).

1.11 Rationale and aims of the study

As early as 300BC, Aristotle commenced his investigation of animal biology and suggested that horses and humans have many biological similarities. Moreover, he tried to characterise many equine respiratory syndromes. Since then, the evolution of veterinary medicine has resulted in the extensive investigation of the pathophysiology of many equine diseases. Nonetheless, hitherto the mechanisms of equine innate immunity are far from completely elucidated.

IAD is one of the most common performance limiting pulmonary disorders in young racehorses in training. Although the precise aetiopathogenesis is poorly understood, proposed mechanisms include opportunistic bacterial infections and/or sub-optimal air-hygiene. Since AMs are the main cells responsible for bacterial and particulate clearance within the lungs, it was hypothesised that they represent an appropriate therapeutic target-cell for the disease. Therefore, acquiring a better understanding of macrophage biology is of paramount importance in addressing this issue. This thesis focuses on the role of AMs in the immune system of the equine lower respiratory tract.

Although all types of tissue macrophages might derive from the same progenitor cells, they develop a function and phenotype that is highly dependent on their milieu. Therefore, the aim of this study was to characterize the function and phenotype of the equine AM and by making comparisons with PMs, to help further define the influential role of the microenvironment.

As mentioned above there is a great demand of animal models that more closely resemble human pathophysiology. Therefore, comparisons are made between the data obtained from horse-derived cells and that published in relation to human, porcine and murine-derived cells, with particular focus on their LPS induced inflammatory status.

Finally, an additional aim was to perform a genome-wide gene expression scan on AMs isolated from Standardbred racehorses prior to and after commencement of competition race training in order to identify any exercise-associated gene expression modulation and to determine whether any mechanisms and biofunctions could be identified which might reflect a training-associated inflammatory response and/or immunological derangement which may increase susceptibility to opportunistic infections.

The next section **(Chapter 2)** describes the methodology followed in this thesis, while **Chapter 3** presents the series of the experiments performed. The findings of this section will be discussed in **Chapter 4**, along with future perspectives of this project. Overall, it will be shown that there is evidence for using horse as an appropriate animal model for human studies, since abundant number of monocytes/macrophages can be isolated from a single individual which, specifically in relation to AMs, show a similar response to LPS. Moreover strong differences between AMs and PMs are presented, highlighting the microenvironmental effect on determining macrophage diversity. Finally, it will be revealed that training, in addition to other factors, may have a significant effect on the respiratory immune system of the horse, specifically in relation to the AM, which represents the first line of cellular immune defence in the lungs.

Together, the information provided here sheds more light on equine macrophage biology, with emphasis on the AM, and provides a first step in understanding its role in IAD, and its potential as a therapeutic target cell. Moreover, the results presented within this thesis form a platform on which future studies can be generated aimed at revealing the role of the macrophage in various other equine and human diseases.

Chapter 2: Materials and methods

2.1 Animals used in the study

2.1.1 Euthanised horses

Eighteen horses [median age: 14.3±1.2 years (mean ± SEM), range: 4-22 years)] were admitted to the Equine Hospital at the Royal (Dick) School of Veterinary Studies for elective euthanasia. The Veterinary Ethical Review Committee of the School of Veterinary Medicine, University of Edinburgh approved all the protocols involving the use of *post-mortem* material from these animals. Horses were euthanised by the intravenous (i.v.) administration of cinchocaine hydrochloride (25mg/mL) and secobarbital sodium (400mg/mL) (Somulose™, Arnolds/Dechra, UK), via a pre-placed 14 gauge i.v. jugular catheter. All animals were systemically healthy and 17/18 had no history of chronic or immediate respiratory disease while one was diagnosed with RAO prior to euthanasia (Horse 7). *Post-mortem* examination confirmed the absence of gross lung pathology in 17/18 horses. One horse had gross evidence of previous EIPH (Horse 4), and another was identified with airway eosinophilia after a differential cell count (DCC) was performed on its BALF (Horse 9).

Samples derived from horses diagnosed with a respiratory condition were not used in the experiments that investigated functional and phenotypic characteristics of the macrophages. The following samples were obtained from these subjects: BALF (n=14), peritoneal lavage fluid (PLF) (n=11), spleen (n=8) and venous blood (n=12). The retrieved samples were immediately placed on ice and processed within 1h of collection at the Roslin Institute. Animals used are detailed in **Table 2.1**.

Horse ID	Age	Sex	Reasons of Euthanasia	Sample
Horse 1	20	F	Elective euthanasia	BALF
Horse 2	15	F	Suspected protein losing enteropathy with poor weight gain-elective euthanasia	BALF, PLF
Horse 3	22	F	Elective euthanasia	BALF, PLF
Horse 4	11	F	Orthopaedic problem- elective euthanasia	BALF, PLF
Horse 5	7	F	Elective euthanasia	BALF, PLF, Spleen
Horse 6	12	F	Elective euthanasia	BALF, PLF, Spleen
Horse 7	10	F	RAO-elective euthanasia	BALF, PLF, Spleen, Blood
Horse 8	4	F	Elective euthanasia	BALF, PLF, Spleen, Blood
Horse 9	13	F	Elective euthanasia	BALF, Spleen, Blood
Horse 10	16	F	Blood bank horse-elective euthanasia	BALF, Spleen, Blood
Horse 11	16	F	Blood bank horse-elective euthanasia	BALF, PLF, Spleen, Blood
Horse 12	16	F	Blood bank horse-elective euthanasia	BALF, PLF, Spleen, Blood
Horse 13	22	M	Blood bank horse-elective euthanasia	BALF, PLF, Blood
Horse 14	22	F	Blood bank horse-elective euthanasia	BALF, PLF, Blood
Horse 15	16	M	Blood bank horse-elective euthanasia	Blood
Horse 16	12	M	Blood bank horse-elective euthanasia	Blood
Horse 17	13	F	Blood bank horse-elective euthanasia	Blood
Horse 18	10	F	Blood bank horse-elective euthanasia	Blood

Table 2.1: Details of euthanised horses.

2.1.2 Standardbred racehorses

Eight Standardbred racehorses (3 males and 5 females) with a mean age: $4.1^{\pm}0.4$ years (mean $^{\pm}$ SEM) and age range: 3-6 years, from a racing yard under the care of the Nantes Veterinary School Equine Clinic were included in this study. The Veterinary Ethical Review Committee of the College of Veterinary Medicine at Nantes approved all the protocols involving the use of these animals. Horses were treated according to standard welfare procedures and informed owner consent obtained. All eight horses were clinically healthy. Clinical examination by two clinicians (Dr Anne Courouce-Malblanc and her resident student Marianne

Depecker) was performed in all the Standardbred racehorses prior to sample collection.

BALF samples were collected at two different time points: prior to the commencement of intense training (T0, end of the rest period) and during a period of training (T1). Horse BU8 was sampled only at timepoint T1. All samples were collected early in the morning between 0600 and 0700, before feeding. The time lapsed between the previous training session and the sample collection was at least 24h. Animals used are detailed in **Table 2.2.** The standard training programme consisted of an initial three week period of light work (jogging), followed by an increase in work intensity to also include two days per week of aerobic exercise, which continued until the blood lactate concentration reached 3-4mmol/L. Apart from horses BU2, BU4 and BU5 which were sampled following an identical 1.5 week period of light work (jogging), the remaining horses had all been in a standardised full training program for a period of 7 weeks. The period between the T0 collection and the initiation of training varied between horses.

Horse ID	Age (years)	Sex	T0 (at rest)	T1 (post rest training period in weeks)
BU1	4	F	2 weeks rest	7 weeks in full training, raced 8 days before sampling
BU2	5	F	6 weeks rest	1.5 week in light training
BU4	5	M	6 weeks rest	1.5 week in light training
BU5	3	F	6 weeks rest	1.5 week in light training
BU6	6	F	6 weeks rest	7 weeks in full training, raced 2 weeks before sampling
BU8	4	M	sampling not available	7 weeks in full training
BU11	3	F	2 weeks rest	7 weeks in full training
BU12	3	M	2 weeks rest	7 weeks in full training

Table 2.2: Details of Standardbred racehorses.

2.2 Sample collection and cell isolation

2.2.1 Bronchoalveolar lavage fluid (BALF)

Post-mortem: Following euthanasia, the trachea was exposed, transected and occluded proximally to prevent blood contamination of the lungs. The thorax was opened and the lungs, with trachea attached, were removed and transferred to a clean working area. The trachea was further transected at a level approximately 15cm from the carina. Two liters of sterile phosphate buffered saline (PBS) (Mg^{2+} Ca^{2+} free; D1408, Sigma-Aldrich, USA) were instilled into the lungs, either directly into the trachea or via a cuffed endotracheal tube. Following gentle massage of the lungs, the BALF was retrieved by gravity following elevation of the lungs above the level of the distal trachea.

Field work: BALF was collected by Dr Anne Courouce-Malblanc and Marianne Depecker as previously described (Depecker et al., 2014). A flexible 3.2 m long, 12.8 mm tip diameter videoendoscope (Optomed) was inserted in the left nostril, guided through the laryngopharynx into the trachea and afterwards introduced into the left or the right mainstem bronchus till it wedged in a more distal bronchus.

A volume of 250mL sterile isotonic saline solution was infused into the bronchus during endoscopy. Initially, the endoscopic channel was filled with 20mL of saline. Afterwards a 125mL bolus of saline was instilled via two 60mL syringes and immediately retrieved by manual aspiration. The initial 20mL of retrieved fluid, representing the dead-space volume of the endoscope biopsy channel, was discarded and the remaining fluid collected. This process was repeated using a further 125mL bolus of saline. Following this procedure, the endoscope instrument channel was flushed with 30mL isotonic saline and the same process repeated in the contralateral lung. The BALF recovered from each lung was transferred separately from metallic bowls into sterile plastic beakers and kept on ice until processed later in the lab. Sedation was required only in horse BU8, which received intravenous administration of romifidine iv (10 mg/l, Sedivet, Boehringer Ingelheim Ltd, St. Joseph, MO, USA).

2.2.2 Peritoneal lavage fluid (PLF)

For *post-mortem* peritoneal lavage, an incision was made in an aseptically prepared area of the ventral abdominal midline down to the level of the *linea alba*, through which a catheter with luminal trocar was inserted into the peritoneal cavity. Following removal of the trocar, 6 L of sterile PBS was infused into the peritoneal cavity by gravity. Following abdominal ballottement, the horse was hoisted by the pelvic limbs and the peritoneal cavity opened carefully. The instilled fluid, which had gravitated to a level immediately caudal to the liver, was visualised and retrieved by siphoning through a 1 m length of sterile tubing.

2.2.3 Cell isolation from BALF and PLF

First the fluid was filtered (100μm cell strainer) and decanted into 500mL falcon tubes and centrifuged at 400g for 10min at 10^0C. Supernatant was removed carefully and the cell pellets re-suspended in PBS and transferred to a 50mL falcon tube which was further centrifuged at 400g for 5min at 10^0C. The cell pellet was resuspended in 1 mL of RPMI-1640 (Sigma-Aldrich, UK) and cells were counted using a haemocytometer. Viability was assessed by adding Trypan blue 0.4%. When grossly visible blood contamination was evident, 5mL of sterile red cells lysis buffer (10mM $KHCO_3$, 155mM NH_4Cl, 0.1mM EDTA pH 8.0) was added for 5min, after which cells were washed once with PBS and centrifuged at 400g for 10min.

2.2.3.1 Differential cell count (DCC) of BALF and PLF

An aliquot of each cell suspension was retained for cytological analysis as described previously (Hughes et al., 2003, Pickles et al., 2002). Cell numbers were adjusted to $5x10^5$ cells/mL by the addition of a calculated volume of PBS. From this aliquot,two cytospin slide preparations were prepared per lavage (cytocentrifuged at 1000g for 3min), stained (Leishman stain; L/1815L/PB05, Fisher Scientific, Leicestershire, UK) and a DCC calculated under light microscopy by counting 500 cells (Hoffman, 1999). Each type of cell was presented as a percentage of total counted nucleated cells. Epithelial cells were not included in the calculations. DCC of the BALF from Standardbred racehorses was performed at the Frank Duncombe Laboratory, in France, by counting 300 cells. Horses were considered free of inflammatory airway

disorders based on the following DCC cut off values: neutrophils ≤ 10%; mast cells <5%; eosinophils ≤ 2% (Hare and Viel, 1998, Koblinger et al., 2011, McGorum and Dixon, 1994, Wasko et al., 2011, Depecker et al., 2014, Richard et al., 2010a).

2.2.4 Spleen cell isolation

A sample (approx. 15g) of horse spleen was collected following euthanasia, immediately transferred into a sterile beaker with PBS and stored on ice. After several washes with PBS to remove peripheral blood contamination, the sample was sectioned into pieces and smashed with a syringe plunger in sterile petri dishes. Spleen tissue was incubated with collagenase (100µg/mL, Sigma C9263-25mg) for 1h in a shaking incubator, following which collagenase activity was inhibited by the addition of EDTA (Sigma E8008, dilution 1:100). The sample was then filtered (100µm cell strainer) and transferred to a sterile 50mL falcon tube. Splenic cells were layered on top of 15mL of Lymphoprep (Axis-Shield, Norway) and centrifuged at 1,100g for 25min with the brake disengaged, following which the distinct layer of separated splenic cells was removed, the cells washed twice with PBS and centrifuged at 600g for 10min and 400g for 10min. Supernatant was discarded and the cell pellet resuspended in 2mL PBS and counted prior to cryopreservation.

2.2.5 Isolation of peripheral blood mononuclear cells (PBMCs)

Blood was collected into sodium heparin vacutainer tubes via a pre-placed 14 gauge i.v. jugular catheter immediately prior to euthanasia. Approximately 80 mL whole blood per horse was collected. After blood was mixed with the same volume of RPMI, 35mL of this suspension was layered on top of 15mL of Lymphoprep (Axis-Shield, Norway) and centrifuged at 1,100g for 25min with the brake disengaged. PBMCs were separated into a distinct layer which could easily be harvested. This was washed twice with PBS and centrifuged at 600g for 10min and 400g for 10min. Supernatant was discarded and the cell pellet resuspended in 2mL PBS and counted prior to use or cryopreservation.

2.2.6 Cryopreservation

Cells not used on the day of sampling were cryopreserved for later use by the gradual addition of freezing medium (FCS 90%, DMSO 10%) (Sigma-Aldrich, USA). Cells were aliquoted at $5x10^7$cells/mL per cryovial. The cryovials were stored overnight in an isopropanol freezing unit at -80^0C (Nalgene, Denmark), facilitating a slow reduction in temperature. The following day, cells were transferred to a -155^0C freezer for long-term storage. When thawing, cells were pre-warmed for 5min in a waterbath at 37^oC. Thawed cells were washed with PBS, added dropwise to minimise osmotic shock. Cells were then centrifuged at 400g for 5min, the supernatant carefully discarded and the cells resuspended in 1mL RPMI for cell counting. Viability of the cells was assessed using Trypan blue 0.4%.

2.3 Cell culture

Cells were seeded in six well plastic plates (Nunc, Thermo Scientific, Wilmington, USA) at $1x10^6$ cells per mL in complete medium: RPMI-1640 medium supplemented with GlutaMAX™-I Supplement (Invitrogen Ltd, Paisley, UK), penicillin/streptomycin (Invitrogen Ltd, Paisley, UK) and 10% heat-inactivated FCS (FCS - PAA Laboratories GmbH, Austria) or 10% heat-inactivated Horse Serum (HS - Sigma Aldrich, cat no: H1138). The response of AMs and PMs cultured in the presence of both horse serum and the more commonly used FCS was compared to further assess whether inter-species differences in macrophage responses to pathogen challenge are due in part to serum proteins, as suggested by Warren *et al* (2010) (Warren et al., 2010). Plates were incubated at 37^0C in 5% CO_2 overnight. The following day, nonadherent cells were removed. New complete medium was added and adherent cells were stimulated with different agonists: LPS (10ng/mL and 100ng/mL) from *Salmonella enterica* serotype Minnesota Re 595 (source strain: ATCC 49284, L9764, Sigma-Aldrich, USA), heat killed *Salmonella enterica; serovar tyhimurium*, strain SL1334 (10^6 cfu/mL, 10^7 cfu/mL and 10^8cfu/mL) and Poly IC (Invitrogen, San Diego, USA) (2µg/mL). Supernatant from the plates was

collected at various time points (0, 2, 6 and 24h) following stimulation and stored at -20^0C until further analysis**.**

Ten million PBMCs were incubated in petri dishes (w/2mm Grid 430196, 60mmx15mm style, Corning Incorporated NY 14831) in complete medium: RPMI-1640 medium supplemented with GlutaMAX™-I Supplement (Invitrogen Ltd, Paisley, UK), penicillin/streptomycin (Invitrogen Ltd, Paisley, UK) and 10% heat-inactivated horse serum (HS - Sigma Aldrich, cat no: H1138), supplemented with recombinant human (rh) CSF1 (1mg/mL) and incubated at 37^0C, 5% CO_2 for six days. Additionally, the same experiment was performed in the absence of rhCSF1 and a higher concentration of HS (30%).

For the endotoxin tolerance experiment, AMs and PBMCs were cultured in the same complete medium as described above in the presence of HS and stimulated with two consecutive LPS (100ng/mL) doses. Twenty four hours following stimulation with the first LPS dose, supernatant was collected, the medium refreshed and cells re-stimulated with the same dose of LPS. Supernatant was collected 24h later and stored at -20^0C until further analysis by enzyme linked immunosorbent assay (ELISA).

2.4 Enzyme linked immunosorbent assay

Equine TNFα and IL10 protein concentrations in cell culture supernatants were measured using the DuoSet ELISA kit (R&D systems, Minneapolis) according to the manufacturer's instructions and a few optimisations. After diluting the capture antibody (Part 841816, 0.4µg/mL for TNFα and Part 842605, 0.8µg/mL for IL10) in PBS, a 96 well microplate was coated with 50µl of capture antibody per well. The plate was sealed and incubated at room temperature overnight. The following morning, the plate was washed three times with 200µl of wash buffer (0.5% Tween^R 20 in PBS, catalog number WA126, R&D systems, Minneapolis). The liquid was carefully removed on each occasion and following the final wash, the plate was turned upside down and tapped on clean papers towels.

Afterwards the plate was blocked with 150µl of Reagent diluent (1% bovine serum albumin in PBS, 0. 2µl filtered, catalog number DY995, R&D systems, Minneapolis) and incubated at room temperature for 1h. Following the wash step, cell culture supernatant samples were diluted in Reagent diluents for TNFα and for IL10 (50µl final dilution). The maximum standard concentrations for TNFα and IL10 were 2000pg/mL and 3000pg/mL, respectively, and a standard curve consisting of seven concentrations prepared by 1:2 serial dilutions in reagent diluent was prepared. The samples were incubated for 2h at room temperature, followed by a repetition of the wash step. Duplicates were assayed for each sample and standard dilution. Control dilutions, called BLANK, were also assayed in duplicate.

Following the wash step, 50µl of diluted Detection antibody (Part 841817, 200ng/mL for TNFα and Part 842607, 100ng/mL for IL10) in reagent diluent were added to each well, the plate was covered and incubated at room temperature for 2h. After the wash step, Streptavidin – HRP (Part 890803, 05% in Reagent diluent) was added and the plate covered to avoid light exposure and left for 20 min at room temperature. Following a final wash step, 100µl of Substrate Solution (1/1 mixture of colour reagent A (H_20_2) and colour reagent B (Tetramethylbenzidine) (catalog number DY999, R&D systems, Minneapolis) were added per well. The plate was kept in the dark for approximately 6 min. Stop Solution (50µl/well, 1N HCl) was then added to discontinue the reaction. Optical density was read on a Multiskan v2.6 (Thermo Scientific, Wilmington, USA) at 450 nm and data analyzed using Ascent software (http://www.selectscience.net/products/thermo-scientific-ascent-software/?prodID=14164&u=30939E35-BD7C-4686-B36E-C532FD388839&techBID=148) .

2.5 Nitrite assay

Nitric oxide (NO) is unstable and is rapidly converted to nitrite. Thus, nitrite was measured as the end product of NO, using the Griess reaction as previously described (Gross and Levi, 1992). Positive and negative controls were obtained by using murine RAW-274.1 cells treated with LPS (10 and 100ng/mL) for 6h, since it is well

established that mouse macrophages produce NO in high levels (Haskó et al., 1996). Duplicates were used for each time point. Briefly, nitrite concentration was assayed against standards by incubating 50µL of supernatant/standard with 50µL Greiss reagent (0.1% α-napthyl-amine, 1% sulfanilimide, 2.1% phosphoric acid) for 10 min. Optical density was read on a Multiskan v2.6 (Thermo Scientific, Wilmington, USA) at 450 nm and data analyzed using Ascent software. Geneious software (http://www.geneious.com) was used to investigate the conservation of the inducible nitric oxide synthetase (iNOs) promoter sequences between human, mouse and horse with a Pustell DNA matrix (Pustell and Kafatos, 1986).

2.6 Flow cytometry assay

2.6.1 CD14, TLR4 and CD163 cell surface expression

Cells were seeded onto cell culture plates at a concentration of 10^6/mL, as previously described **(Section 2.3)**, and left overnight. The following morning nonadherent cells were washed away and remaining adherent cells were gently detached using cell scrapers. One million cells were used per vial. After 30 min incubation at 4^0C in the dark with high-block solution (PBS, 0.1% NaN3, 2% FCS, 0.1%BSA), cells were washed with low-block solution (PBS, 0.1% NaN3, 0.2% FCS, 0.1%BSA) and centrifuged twice at 400g for 5min.

Cells were then incubated for 30 min in the dark with 100µl of low-block solution and stained with either mouse monoclonal anti-equine CD14 IgG1 Ab, goat polyclonal anti-human TLR4 antibody or a mouse anti-human macrophage surface Ag monoclonal antibody targeting CD163. After three washes with low-block solution and centrifugation at 400g for 5 min, a F(ab') 2 goat anti-mouse secondary IgG was used for the CD14 and CD163 Ab staining, while a donkey secondary anti-goat (Alexa-Fluor 633, Life A21082) was used for the TLR4 staining in 100µl of low-block solution. The cells were incubated for 30 min at 4^0C in the dark **(Table 2.3)**. Cells were then washed, resuspended in 500µl of PBS and analysed using

CyAn™ ADP Analyser (Beckman Coulter, **High Wycombe, UK)** and Summit v4.3 software (http://summit1.software.informer.com/4.3/). Sytox Blue 0.1% (Invitrogen, Catalogue No S34857) was added to exclude any dead cells from analysis. Non-specific binding was controlled by using the secondary antibody only and all centrifugation steps were performed at 4^0C. Data were acquired on approximately 10,000 events.

Antibody	Concentration	Source
Mouse anti-equine CD14	1:100	Clone 105, Wagner Laboratory, Cornell University
Goat anti-human TLR4	1:100	M-16:sc-12511, Santa Cruz Biotechnology, Inc
Mouse anti-human CD163 (AM-3K)	1:100	KT013, Gentaur
Goat anti-mouse IgG F(ab') 2	1:100	SATR105PE, RPE AbD Serotec,
Donkey anti-goat IgG	1:200	Life A21082, Alexa-Fluor 633,

Table 2.3: Antibodies used in flow cytometry.

2.6.2 Phagocytosis assay

Cells were seeded in duplicate ($1x10^6$cells/mL), in petri dishes (w/2mm Grid 430196, 60mmx15mm style, Corning Incorporated NY 14831) in complete medium (supplemented only with HS) and left overnight at 37^oC, 5% CO_2. The following morning, two plates were incubated at 4^0C for at least 30min. The 4^0C condition was incorporated to reveal the presence of surface adherent bacteria only, as phagocytosis is inhibited at this temperature. Medium from all plates was refreshed with warm and

cold cell culture media (as above), respectively; thus, removing nonadherent cells. Heat-killed *Escherichia(E.) coli* bioparticles (E2861, K-12 strain, 10mg, Life techonogies, Molecular probes^{R}) were added at a ratio of 10 bacteria per cell in one plate at 37^{0}C and one at 4^{0}C. No bacteria were added to the remaining 4^{o}C and 37^{o}C plates; these were incorporated to control for autofluorescence. Paired plates were incubated for 1h at 4^{o}C and 37^{o}C, respectively, to allow particle uptake. All cells were washed twice with cold PBS and were gently collected with cell scrapers. Cells were then centrifuged at 400g for 5min, resuspended in 500 mL of cold PBS and analysed on a CyAn™ ADP Analyser, as above.

2.7 RNA analysis

2.7.1 Total RNA extraction

Total RNA from both AMs and PMs was extracted using RNA-Bee (Amsbio, Abingdon, UK) according to the manufacturer's instructions. After carefully removing the supernatant of the cell culture plates, 1mL of RNA-Bee was added for the cell lysis, since at least 0.2 mL of RNA-Bee per 10^{6} cells was indicated by the protocol. Then 0.2 mL chloroform was added per 1 mL of RNA-Bee. The sample was shaken vigorously for approximately 30 sec and left on ice for 5 min.

The homogenate was centrifuged at 12,000g for 15 min at 4^{0}C. Afterwards, the sample formed a lower blue phenol-chloroform phase, an interphase, and an upper colourless aqueous phase. The aqueous phase contained the RNA and had almost 50% of the volume of the RNA Bee used, plus the volume of the sample. After transferring it to a clean tube for the precipitation step, 0.5 mL of isopropanol was added, the sample stored for 30 min at room temperature and then centrifuged at 12,000g for 15 min at 4^{0}C.

Following centrifugation, the supernatant was removed, the RNA pellet washed once with 75% ethanol and then centrifuged for 10 min at 7,500g at 4^{0}C. At least 1 mL of

ethanol solution was used per 1 mL of RNA-Bee used for the initial homogenization. Finally, the RNA pellets were air-dried for 10 min, then dissolved in 50 µl of RNase-free water and incubated for 10 min at 55-60^{0}C.

2.7.2 RNA quality assessment

RNA concentration and purity was measured using ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) by measuring absorbance at 260 and 280 nm (A260, and A280 respectively). Purity of RNA was determined using the A260/A280 ratio. A ratio close to two is indicative of pure RNA. RNA integrity was confirmed with RNA 6000 Pico Assay (Agilent Technologies). RNA integrity number (RIN) greater than seven as considered appropriate for qPCR and microarray use (Schroeder et al., 2006).

2.7.3 cDNA synthesis

One microgram total RNA was converted to complementary DNA (cDNA) using the NanoScript reverse transcription kit (RT –NanoScript, Primerdesign, Southampton), according to the manufacturer's instructions. A 10µl final solution contained 1µg of RNA, 1µl of random nonamer primer and RNAase/DNAase free water. Solutions were heated for 5min at 65^{0}C and then stored on ice. The transcription mix contained 2µl of nanoScript 10x Buffer, 1µl of 10mM dNTP, 2µl of 100mM DTT, 1µl of NanoScript enzyme and 4µl RNAse/DNAse free water to make a final volume of 10µl. 10µl of the transcription mix was added on each of the samples stored on ice. Negative controls were included by omitting the NanoScript enzyme in order to check for genomic contamination (NRT control). The samples were incubated for 5 min at room temperature, then for 20 min at 55^{0}C and finally at 75^{0}C for 15 min. cDNA was stored at -20^{0}C until further use in the RT qPCR reaction.

2.8 Real time quantitative polymerase chain reaction (RT qPCR)

All the RT qPCR reactions were carried out using the Stratagene Mx3000p qPCR System following the manufacturer's instructions. RT qPCR with SYBR Green chemistry was performed according to the custom designed real time PCR assay from Primerdesign. Each sample was tested in triplicate. A negative control, containing no reverse transcriptase, produced during cDNA synthesis (NRT control) **(Section 2.7.3)**, and a second negative control containing no cDNA (NT control), which reveals possible contamination problems, were always included in each assay. Primers for the genes of interest were provided by Primerdesign. Primer details are shown in **Table 2.4**. *18S ribosomal RNA* (*18S*) was selected as a housekeeping gene according to the results of the GeNorm kit used in the current study **(Section 2.8.1)**.

The cDNA of the samples (LPS treated and untreated) was diluted in RNA/DNAase free water to a concentration of 5ng/mL. Five microliters of each sample was added to 1µl primer mix, 10µl Primerdesign Precision SYBRgreen 2X qPCR Mastermix, 0.5µl ROX reference dye (Invitrogen, Catalogue No 12223-012) and 3.5µl RNA/DNAase free water, in order to make a final volume of 20µl. RT qPCR cycling conditions are shown on **Table 2.5**.

Primer Name	Sequence (5'-3')	Tm (^{0}C)	Product Length
***TNFα* sense**	ATCTACTCCCAGGTCCTCTTC	55.9	129
***TNFα* antisense**	CGTGTTGGCAAGGCTCTT	55.7	
***IDO* sense**	ATCAAAGAAATTCCGTTATATTCAA	54.1	94
***IDO* antisense**	TGCGTAGACAAGAAGAAGTTATATCAAT	55.6	
***STAT 4* sense**	TCAGTAGAATTTCGCCATTTGC	56.2	119
***STAT 4* antisense**	ATCTGTGTCTCAAAGGTTATGGA	55.9	
***IL7R* sense**	TCCTCCGCAACTAGATGATTC	55.5	109
***IL7R* antisense**	CTCTCCTCCGAAAGTTTTTGG	55.1	
***CYP27B1* sense**	AGTTGTCAGCCTCGTCCAT	56.3	96
***CYP27B1* antisense**	GTTCATACAGCCCAAGAGAG	56.7	
***TNIP3* sense**	CATGGCACATTTTGGACAGG	55.7	95
***TNIP3* antisense**	AGTTCTTTCTTCTTGATGATTGAGT	55.8	
***18S* sense**	CGGACAGGATTGACAGATTGATA	56.8	118
***18S* antisense**	TGCCAGAGTCTCGTTCGTTA	56.8	
***ACTB* sense**	GGCATCCTGACCCTCAAGT	56.7	194
***ACTB* antisense**	GCTCGTTGTAGAAGGTGTGG	56.6	
***B2M* sense**	AGGACTGGTCTTTCTATCTTCTG	55.8	111
***B2M* antisense**	ACTTAACTATCAGGGGGTCTTTG	56.3	
***GAPDH* sense**	GGAGTCCACTGGTGTCTTCA	56.9	91
GAPDH antisense	AGCAGAAGGAGCAGAGATGAT	56.8	
***SDHA* sense**	GGACAGAGCCTCAAGTTTGG	56.5	116
***SDHA* antisense**	GTATCATATCGTAGAGACCTTCCATA	56.6	
***HPRT1* sense**	CTGAGGATTTGGAAAAGGTGTTTAT	56.9	89
***HPRT1* antisense**	TCCCATCTCCTTCATCACATCT	56.8	

Table 2.4: Oligonucleotides used in RT qPCR analysis

Step	*Time*	*Temperature*	*Number of cycles*
Enzyme activation	10 min	95^0C	1
Denaturation	15 sec	95^0C	40
Annealing and extension	1 min	60^0C	
Melt Curve	1 min	95^0C	1
	30 sec	60^0C	
	15 sec	95^0C	
	30 sec	25^0C	

Table 2.5: RT qPCR reaction thermal profile

Real time qPCR data were analysed with the Stratagene MxPro qPCR Software according the standard curve method as previously described (Winer et al., 1999). Expression level values were first derived from the standard curve equations and were normalised to the reference gene *18S rRNA*, as described in **Section 2.8.2** by applying the following formula:

$$relative\ gene\ expression = \frac{average\ of\ target\ gene}{average\ of\ reference\ gene}$$

2.8.1 Primer efficiency

The efficiency of the primers was determined by analysing standard curves for each set of primers. Standard curves were run for each RT qPCR plate. Examples are presented in **Appendix I**. Five serial two fold dilutions of cDNA reaction mixtures

from 1:10 to 1:160 were made and RT qPCR reactions were performed in triplicate for each primer mix separately.

The cycling threshold (Ct) values of each of the dilutions were plotted against the cDNA dilutions and the slopes calculated from the graphs. Graphs with a high correlation coefficient (Pearson's correlation coefficient $r \geq 0.90$) and high linearity in the investigative range are representative of high primer efficiency. One hundred percent efficiency corresponds to a perfect doubling of template during every cycle, however, the conventional range is 90-110% for assay validation, which corresponds to standard curve slopes of -3.1 to -3.6.

2.8.2 Reference gene selection

The establishment of the optimal reference genes was performed by using the geNorm^{TM} reference gene selection kit (Primerdesign), according to manufacturer's instructions. The genes tested were the *18S*, *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *Beta-actin* (*ACTB*), *β2 microglobulin* (*B2M*), *Succinate dehydrogenase complex, subunit A* (*SDHA*) and *Hypoxanthine-guanine phosphoribosyltransferase* (*HPRT1*).

In order to select an appropriate reference gene, cDNA of the samples (4 LPS treated and 4 untreated) was diluted in RNAse/DNAse free water to a concentration of 5ng/mL and RT qPCR assay was performed as described above **(Section 2.8)**. qBase^{PLUS}, a real time data analysis software, was used for the analysis of the results. Genorm M and V analysis was performed (http://www.biogazelle.com/). The M value indicates the average expression stability value of the reference genes; the lower it is, the more stable the gene. The V value determines the optimal number of reference genes required in each experiment by illustrating the variation in average stability of the reference genes. This calculation is known as pairwise variation, V(n/n+1). The threshold of the V value is 0.15.

2.9 Statistical analysis

Descriptive statistics were performed using Mini Tab 16 statistical software (Minitab Ltd., UK). After testing for normality, a one-sample Wilcoxon test was employed to identify differences in protein production/gene expression production between (a) untreated cells (controls) and cells incubated for 2h, 6h, 24h following multiple antigen challenge stimulation, (b) cells incubated with FCS and HS, (c) low and high dose of endotoxin (d) fresh and cryopreserved cells and (e) before [T0] and during the training period [T1], and to compare the DCC between timepoints T0 and T1. A Mann-Whitney test was performed to identify any statistical significance in the protein production between AMs and PMs and to compare the DCC findings between the racehorse and nonracehorse groups. A two sample t-test was performed to identify any statistically significant differences in the BALF DCCs between both healthy and diseased horses and racehorses and nonracehorses. Statistical significance was assumed at $p<0.05$. Numeric results are presented as mean$^{\pm}$ SEM. All the experiments were performed using different biological replicates.

2.10 Microarray analysis

Microarrays were processed by ARK Genomics (Centre for Comparative and Functional Genomics) at the Roslin Institute. Total RNA (50ng) was amplified by the Nugen Pico SL kit (Agilent, the Netherlands). Two and a half micrograms (2.5µg) of the cDNA produced was biotin labelled using the Nugen Encore labelling kit. Biotin labelled transcripts were prepared for hybridisation following the Nugen protocol for Gene Titan hybridisation (Affymetrix, Santa Clara, CA), using the Affymetrix Gene Titan Hybridization Wash and Stain Kit for WT Array Plates (PN 901622). The samples were hybridised to Equine Gene 1.1 and 1.0 ST Array Strips from Affymetrix, including 30,559 probe sets, using the appropriate Hyb-Wash Scan protocol and the Gene Titan Hyb-Wash Stain Kit for the reagents (Affymetrix, Santa Clara, CA). The arrays used were oligonucleotide microarrays that consisted of probes corresponding to exons along the whole length of each transcript (25 probes sets per transcript). However, 41.3% of the transcripts included in the arrays were

missing gene symbol information, due to the poor annotation of the horse genome. The microarrays also included a small number of duplicate transcripts (183 out of 17,929 annotated transcripts), that correlated during the analyses.

Image preparation and the CEL files required for the analysis were produced using Affymetrix Gene Chip Command Console Software (AGCC) version 3.0.1. For the quality control, Affymetrix Expression Console Software was used. Expression values were normalized according to the RMA algorithm within the Expression Console Software (Irizarry et al., 2003). Equine Gene 1.1 ST Affymetrix Array Strip (Affymetrix, Santa Clara, CA) was used for the first batch of horses (horses 1-3, LPS treated and controls). After this, due to lack of availability of this strip from Affymetrix, Equine Gene 1.0 ST Affymetrix Array Strip was used for the remaining samples. Comparisons between the two array strips were feasible since the same group of probesets (30,559 probesets) was used in both array types.

2.10.1 Partek microarray analysis

CEL files were imported to Partek Genomic Suite 6.6 software (North America Partek Incorporated, St. Louis, Missouri) for the microarray data analysis, using default parameters. The resulting data were expressed in Log2. Principal-component analysis (PCA) was performed to detect outliers and assess whether batch effects or other parameters significantly affected the data.

- A two way ANOVA (random effect: array type; fixed effect: cell type) was used for the microarray analysis to compare untreated AMs and PMs in the **Section 3.2.6**. In this case the gene list was created by using an adjusted p value <0.01 and a fold change of 9.
- Data from untreated AMs at 0h were compared with the LPS treated AMs at the 6h time point **(Section 3.3)**. A three way ANOVA (random effect: array type and horse ID; fixed effect: LPS treatment) was executed to compare differential expression in LPS treated and untreated AMs. A gene list was

created with a cut off adjusted p value <0.05 and a fold change of two for the LPS treated AMs.

- The same statistical analysis was performed in **Section 3.3.1**, however in that case the array type was not included in the analysis, since only one type of arrays was utilised for that experiment. The microarray datasets used here derived from a human Reynier *et al* (2012) study (GDS4419, platform: GPL570: [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array).
- In **Section 3.4.3** a two way ANOVA (time point: fixed effect; horse ID: random effect) test was performed in order to identify differentially expressed genes between the two groups of standardbred racehorses at rest and during the training period ($p< 0.05$, fold change of two).
- In the final experiment described in the **Section 3.4.4**, a two way ANOVA with horse type as a fixed effect and scan date as a random effect, statistical significance 0.05 and fold change of two was performed in order to detect any differentially expressed genes between young racehorses and elderly nonracehorses.

FDR (False discovery rate) or adjusted p-value using the Benjamini and Hochberg test for correction was used in all the experiments. Fold changes of at least two were used in all the experiments. These cutoff values are widely used in microarray data analysis. The only case in which more strict values were applied was in the comparison between AMs and PMs. In that case the vast difference between these two groups resulted in a very long gene list, that allowed the use of more firm cutoff points.Upregulated transcripts were represented with positive values, whereas negative values were used for the downregulated transcripts.For the validation of the microarray results, RT qPCR was performed in the same samples utilised for the microarray experiments. The same protocol as described in **Section 2.8** was followed to target sequences of the genes of interest.

2.10.2 Biolayout microarray analysis

A network analysis of the expression data was performed in BioLayout *Express*^{3D} (Freeman et al., 2007). With this software pairwise Pearson correlation coefficients were calculated and a threshold of r>0.9 was chosen for the transcript to transcript comparison across the array samples. The resulting network graph consists of nodes that represent transcripts and the edges that represent expression correlations above the threshold (r>0.9) between the transcripts. The markov cluster algorithm (MCL) graph based algorithm was used to identify groups of tightly co-expressed genes, with an inflation value of 2.2 (van Dongen and Abreu-Goodger, 2012). Lowly expressed transcripts, with dynamic range ≤1.5, were filtered out.

2.10.3 Functional annotation

Initial annotation used the most recent Affymetrix annotation file imported in Partek software or identified in the Affymetrix Netaffx site and additional annotation was achieved by cross-mapping the non-annotated sequences to the human sequence using BLAT in the Ensembl browser (Couture et al., 2009, www.affymetrix.com).

DAVID (Database for Annotation, Visualisation, and Integrated Discovery) 6.7 software, as well as Partek Genomic Suite 6.6 software were used to determine the biological processes of the genes included in the gene lists. The enrichment score of the DAVID package is a modified Fisher exact p value calculated by the software, while in Partek software it is measured by a chi-square test comparing the proportion of the genelist in a group to the proportion of the background in the group tested. The higher the enrichment score is, the more enriched the cluster is. An enrichment score of greater than one means that the functional category is over expressed. A value of three corresponds to significant over expression (p-value of less than 0.05).

Chapter 3: Extensive studies on the equine macrophage/monocyte

The overall goal of the current study was to characterise the equine monocytes/ macrophages with a view to understanding their functions in homeostasis and pathology, as well as comparing them with macrophages from other, better characterised, mammalian species such as human, mouse and pig.

3.1 Establishing a protocol for horse macrophage isolation and activation

The horse is a valued and valuable animal, so it was important to achieve maximum utility from each animal euthanised. Therefore the first stage of this project was to establish the conditions for isolation, preservation, culture and stimulation of macrophages from different locations in the horse.

3.1.1 Monocytes / macrophages can be isolated in high yield from the lungs, peritoneal cavity, spleen and peripheral blood from a single horse.

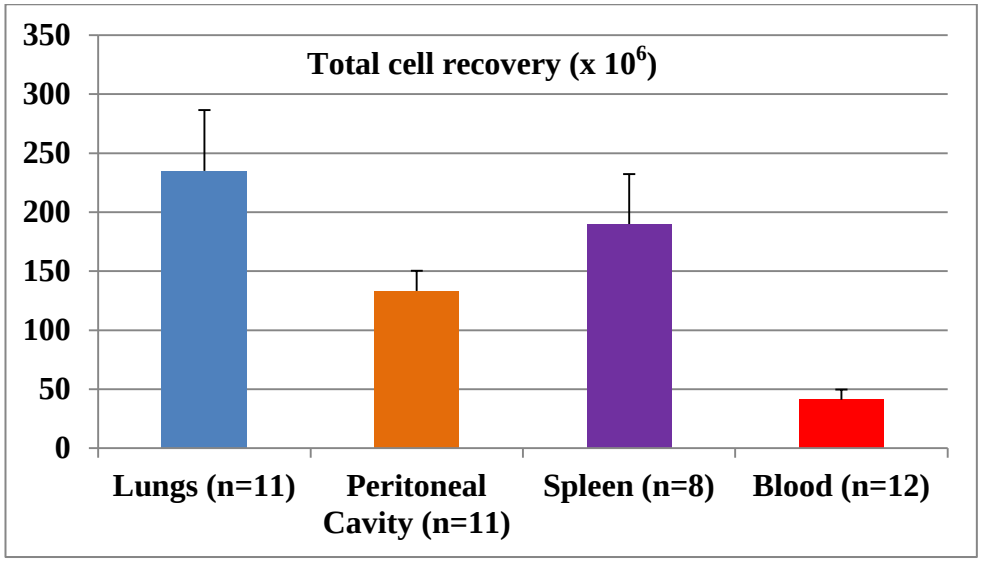
Sample collection from different anatomical sites is essential not only for disease diagnosis and health status assessment, but also for research purposes, both in human and veterinary medicine (Hoffman, 1999, Mezhir et al., 2013, Zhang and Morrison, 1993). In particular, BALF, peritoneal fluid and blood samples are routinely used for diagnostic purposes by equine veterinarians (Hoffman, 1999, Harrold et al., 2000, Richard et al., 2010b). Contrary to mice, where small volumes (*circa* 10mL) of blood and lung and PLF can be isolated from a single animal (Zhao et al., 2005), horses represent an abundant source of different types of immune cells. Isolation protocols for equine macrophages/monocytes from blood, lungs and peritoneal cavity of live horses have been reported (Laan et al., 2005, Morris and Moore, 1987, Raabe

et al., 1998) while one study on *post-mortem* on BALF retrieval in an abattoir is published (Hammond et al., 1999a).

However, a greater volume of BALF and PLF, and consequently cell yield, can be obtained from horses following euthanasia. For example 75-250mL of PLF per horse can be obtained from live horses (Hawkins et al., 1998, Massoco and Palermo-Neto, 2003) , compared to 1.5-3L per horse obtained following euthanasia. Similarly, *circa* 250-300mL is routinely instilled into the bronchus of a live horse (Lavoie et al., 2011, Laan et al., 2006), while in the current study a volume of 2L was instilled into the trachea. These volumes and associated cell retrieval values represent 2-3 orders of magnitude greater than can be obtained from rodent models.

The present study used a series of 18 horses euthanised for a variety of reasons **(Table 2.1)**, and an average of 243 $x10^6$ ($\pm$49 $x10^6$) cells were isolated from the lungs, 125 x 10^6 ($\pm$13) from the peritoneal cavity, 189$x10^6$ ($\pm$42) from the spleen (approximately 15g) and 41$x10^6$ ($\pm$9) from the peripheral blood (approximately 80ml) **(Figure 3.1.1.A)**. A greater cell yield was obtained from the lung compared with peritoneal cavity. Although this finding may reflect the relatively greater surface area of tissue lavaged, it may also reflect a difference in cell density (per unit surface area) between these anatomical sites. Due to the extensive lung surface area, which is continuously exposed to numerous inhaled pathogens and pro-inflammatory agents; there is likely a greater requirement for an abundance of immune cells in the lungs compared to the peritoneal cavity. The yield of cells from lungs of horses with evidence of pulmonary disease was statistically significantly lower than that from healthy individuals **(Figure 3.1.1.B)**, potentially due to airway narrowing and fluid retention in the distal airways. In the cadaver, this airway narrowing may be due to disease-associated mild structural changes as previously reported in horses affected with EIPH (McKane and Slocombe, 2002), as opposed to bronchospasm resulting from airway hyper-responsiveness in the live horse, a mechanism cited as the cause of low fluid retrieval in horses with a high percentage of metachromatic cells (Lavoie et al., 2011).

A



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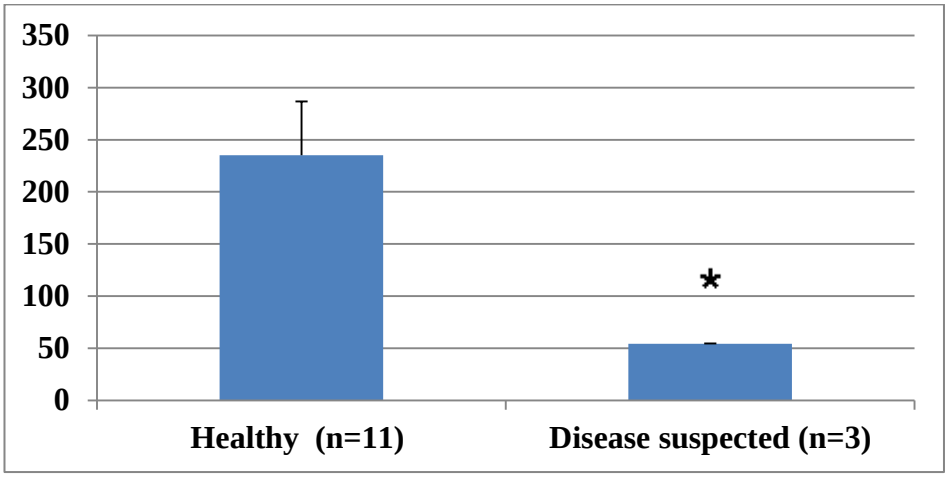


Figure 3.1.1: Cells harvested from different anatomical regions of the horse and from the lungs of horses with and without respiratory disease.

Total cell recovery ($x10^6$) from ***(A)*** *the lungs, peritoneal cavity, spleen and blood of healthy horses and* ***(B)*** *the lungs of horses with and without a suspected pulmonary inflammatory disorder. Results are presented with the mean +/- SEM (*p<0.05 versus healthy horses).*

3.1.2 Cryopreserved monocytes/macrophages can be recovered from storage and retain their activity

Given the high yield of cells, there are obvious economical and ethical benefits to harvesting and storing homogenous macrophage populations from a single donor, especially since this allows the cells to be used for repeated experiments incorporating multiple time points. The production and maintenance of cell lines has been proposed as one solution to this problem (Werners et al., 2004). However, continuous subculture may result in important genetic or epigenetic changes, yielding cell populations that do not represent their *in vivo* counterpart (Ho-Yeon et al., 2007). Several cryopreservation protocols utilizing different cryopreservatives, storage temperatures or containers have been tested for different animal species and cell types (Berz et al., 2007, Marim et al., 2010, Kapetanovic et al., 2012). In the current study, the widely used protocol of controlled freezing in DMSO (10%) and FCS (90%) was used for cell cryopreservation and cells were stored at -155^{0}C (Kapetanovic et al., 2012, Marim et al., 2010). Similar cryopreservation protocols have been used elsewhere for equine AMs, bone marrow monocytes, spleen macrophages and stem cells, but not for PMs (Werners et al., 2004, Martinello et al., 2010, Moore et al., 2003, Raabe et al., 1998).

Cell viability on the day of cell harvesting was greater than 90% as assessed by Trypan blue staining. Short-term (*circa* 1 month) and long-term (*circa* 6 month) cryopreservation caused little if any loss of cell viability, which remained higher than 80% in all cases **(Figure 3.1.2)**. This showed that cells from a single horse could be harvested, cryopreserved and stored for later use.

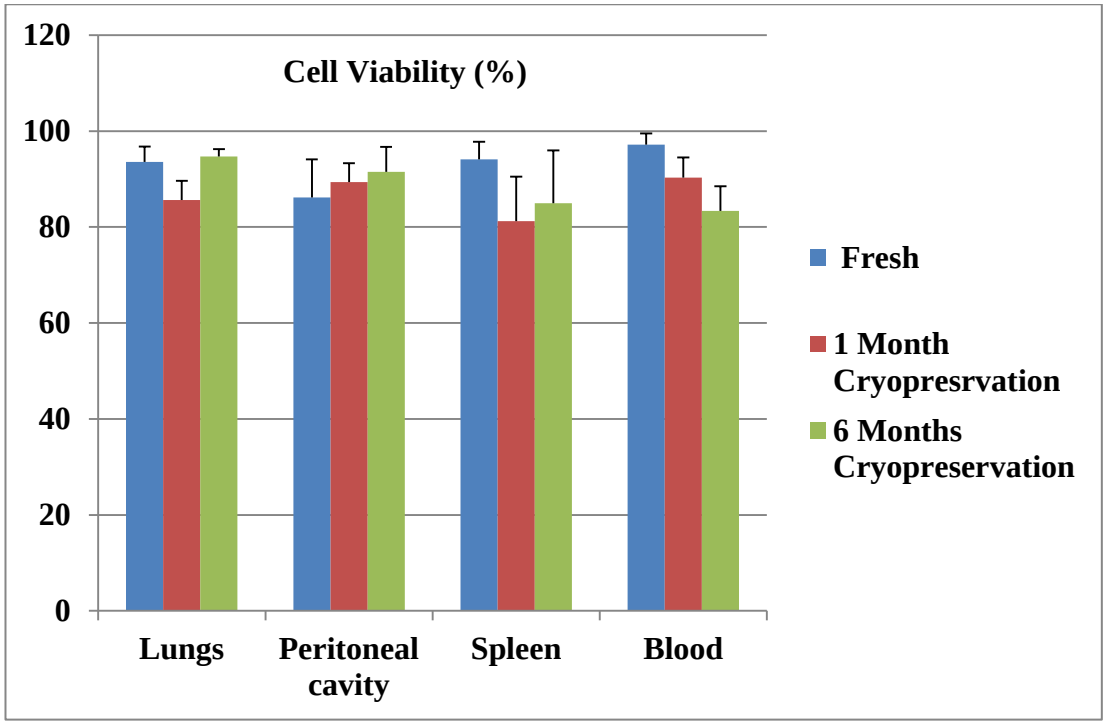


Figure 3.1.2: Cell viability.

Cell viability (% live cells) was assessed using Trypan blue staining on fresh and cryopreserved (1 month or 6 month storage) cells after thawing. Results are the mean of a minimum of 8 experiments +/- SEM.

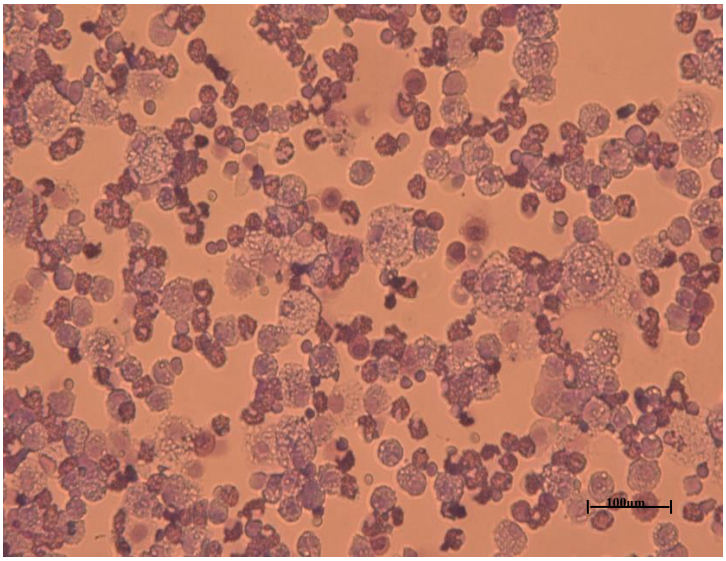
3.1.3 Differential cell counts on alveolar and peritoneal lavage

The DCC in BALF and PLF provides useful information for the diagnosis of diseases of the lungs and abdomen (Hoffman, 1999, Barton et al., 1996). Widely, reported DCC reference ranges in BALF are: 30-60% AMs, 30-70% lymphocytes, 0-5% neutrophils, 0.6-2% mast cells, 0-1.6% eosinophils (Mair et al., 1987, Moore et al., 1995, Mazan et al., 2005, Couetil and Denicola, 1999), although others reported a greater number of mast cells, eosinophils or neutrophils in the BALF of healthy horses (Derksen et al., 1985, McGorum and Dixon, 1994, Richard et al., 2010b, Richard et al., 2010a). Reported differences in mast cell differential counts may be technical in origin, related to the degree of mast cell degranulation and the staining

technique used. Occasionally, gross blood contamination was observed in the PLF, making it difficult to assess the DCC by light microscopy.

Results for DCCs of *ex vivo* BALF and PLF from healthy horses, as assessed by light microscopy **(Figure 3.1.3)**, are summarized in **Table 3.1.1** which shows that that PLF consistently yielded a lower proportion of macrophages and lymphocytes and higher proportion of neutrophils than BALF. The DCC of the three horses suspected as having a respiratory disorder revealed significant increases beyond the reference ranges in the percentages of haemosiderophages and granulocytes, which indicate the presence of respiratory disease (**Table 3.1.2**). The BALF cytology profile of the horse diagnosed with RAO was similar to that already reported in such cases and cytological analysis also revealed evidence of airway disease (eosinophilia and increased haemosiderophages) in two other horses without any preceding history of respiratory signs (Couetil et al., 2005, Bell et al., 2008, Michelotto Jr et al., 2011, Richard et al., 2010a, Hare and Viel, 1998). The horse with the increased haemosiderophages was a racehorse and this cytological finding was considered consistent with a diagnosis of EIPH.

A



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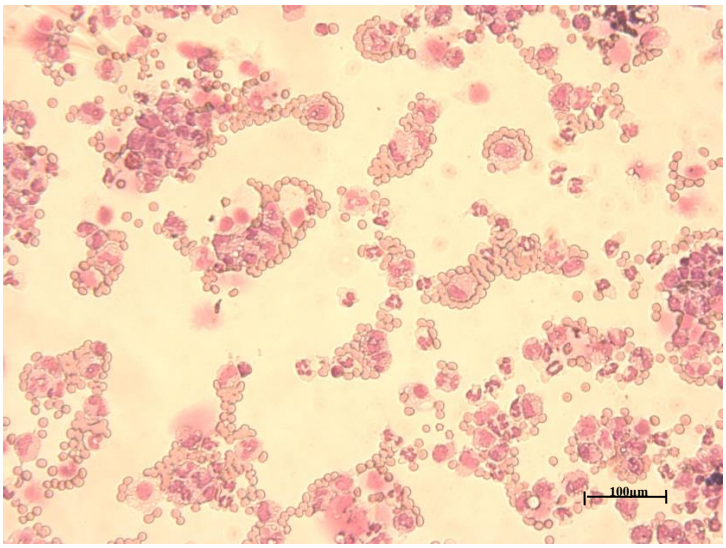


Figure 3.1.3: Photomicrographs of stained cytospin preparations of BALF and PLF.

Cell numbers from the BALF ***(A)*** *and PLF* ***(B)*** *were adjusted to 5x10^{5}cells/mL by the addition of a calculated volume of PBS. Numerous erythrocytes surround the PMs in the PLF. From this aliquot, 2 cytospin slide preparations were prepared per lavage sample (1000g for 3min), stained with Leishman stain and a differential cell count determined under light microscopy by counting 500 cells. Figures show light microcopy images. Scale bar = 100µm.*

	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Mast cells (%)	Eosinophils (%)
Bronchoalveolar Lavage (n=11)	***70.8 (±2.4)***	***22.3(±2.2)***	***2.7(±1.1)***	***3.6(±0.9)***	***0.63(±0.4)***
Peritoneal Lavage (n=11)	***55.9(±6)***	***7.2(±2)***	***36.2 (±6)***	***0.7(±0)***	**-**

Table 3.1.1: DCCs (mean±SEM %) of BALF and PLF derived from healthy horses

Horse ID	Condition	Macrophages (%)	Haemosiderophages (%)	Lymphocytes (%)	Neutrophils (%)	Mast cells (%)	Eosinophils (%)
Horse 4	***Exercise induced pulmonary haemorrhage***	***45***	***26.3***	***23.3***	***5.1***	***0.3***	**-**
Horse 7	***Recurrent airway obstruction***	***30.3***	**-**	***22.6***	***44.9***	***2.2***	**-**
Horse 9	***Significant airway eosinophilia***	***45.5***	**-**	***14.5***	***3.5***	***1.5***	***35***

Table 3.1.2: DCCs (%) of BALF derived from horses (n=3) suspected as having a respiratory disorder.

In order to confirm the differential counts calculated by light microscopy, cells from BALF were stained for CD14, the co-receptor for LPS, usually expressed on macrophages **(Figure 3.1.4)** (Kabithe et al., 2010). As discussed in the introduction, **Section 1.2**, CD14 is a co-receptor for the complex of LPS, LBP and TLR4 and is identified on the surface of cells of the MPS.

The 60-70% of cells that showed high expression of CD14 ($CD14^{hi}$) also showed greater side scatter during flow cytometric analysis, consistent with a population of macrophages (Region 1, R1), whereas the smaller and less granular CD14-negative cells were consistent with lymphocytes (Region 3, R3, 20-30%). The remaining cells (Region 2, R2), which were granular and showed low expression of CD14 ($CD14^{low}$), were likely a mixture of neutrophils, mast cells and monocytes.

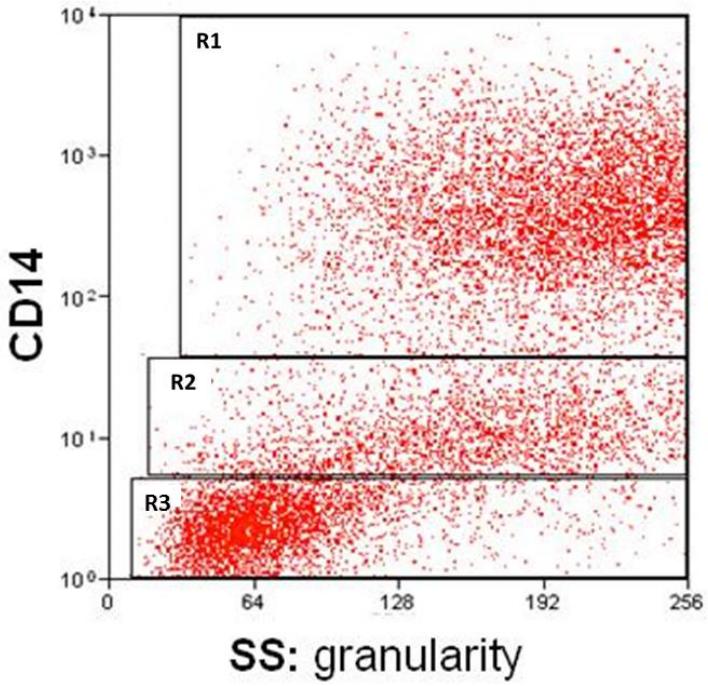


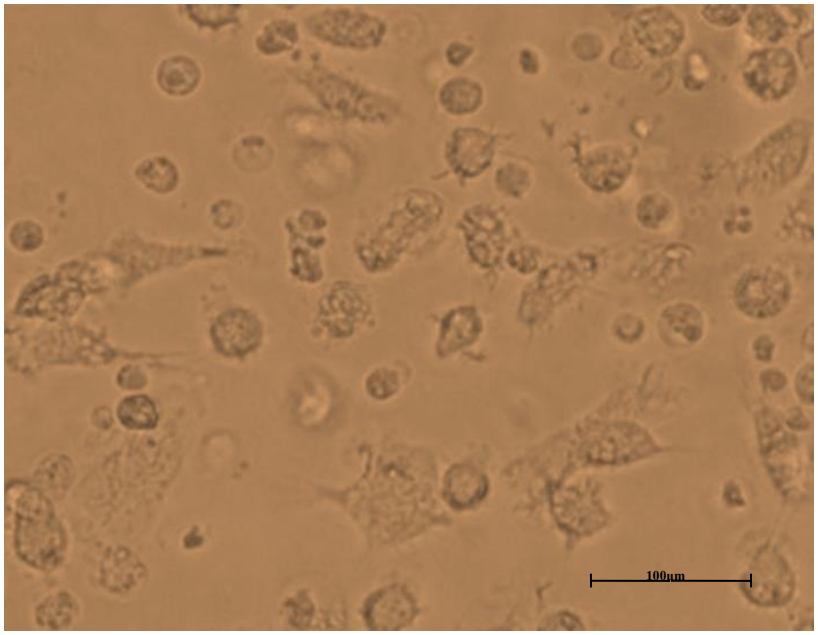
Figure 3.1.4: Flowcytometric characterization of the harvested BALF cells.

BALF cells were stained with CD14 antibody. Median fluorescence intensity and granularity were measured by flow cytometry, using a total of 10,000 events. Region 1 (R1) represents cells with high expression of CD14 and high granularity (macrophages), R2 represents cells with low CD14 and high granularity (mix of monocyte/neutrophils) and R3 represents cells with no granules and no CD14 (lymphocytes). These data are representative of three experiments.

3.1.4 Alveolar macrophages differ from peritoneal macrophages with respect to their morphology

Macrophages of the lung have a distinct cellular phenotype in different species such as pigs, humans and mice, a finding considered to reflect the distinctive microenvironment of this cell and its "breath-by-breath" exposure to ambient air containing a variety of potential allergenic and pro-inflammatory components (Guth et al., 2009, Freeman et al., 2012, Ettensohn and Roberts, 1983). Following cryopreservation, both equine PMs and AMs retained a phagocyte-like morphology, including granularity and pseudopodia. There were clear differences in the cell morphology between AMs and PMs cultured in either FCS or HS. AMs were larger in size, had a greater number of granules within their cytoplasm and developed a greater number of pseudopodia compared to PMs **(Figure 3.1.5)**. As shown earlier (Laan et al., 2005), following overnight culture and removal of nonadherent cells, more than 85% of adherent cells were identified morphologically as macrophages after performing a Leishman stain on the culture plates, for both AMs and PMs **(Figure 3.1.6)**. The potential for minimal lymphocyte contamination could not entirely be excluded. However, such an occurrence was considered highly unlikely to affect the data derived from studies designed to investigate predominantly macrophage-specific functions.

A



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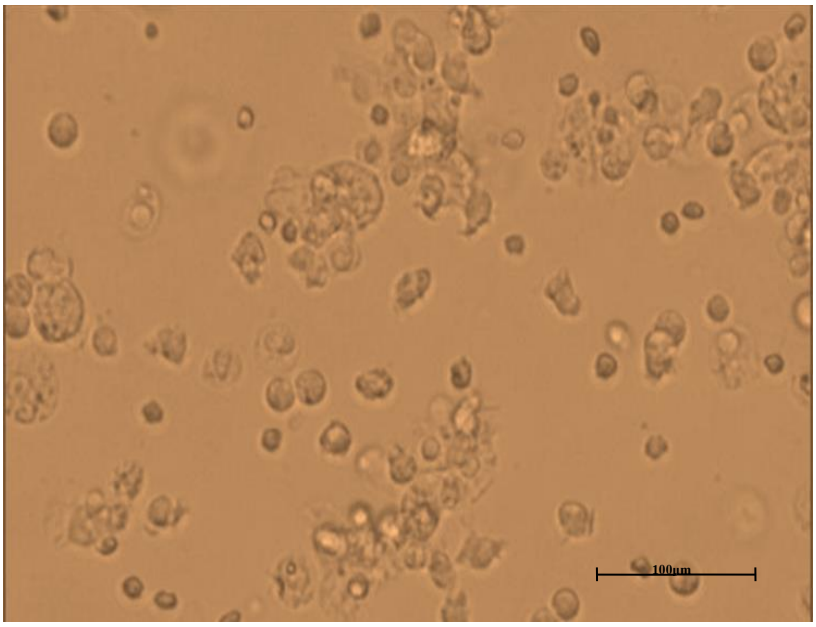
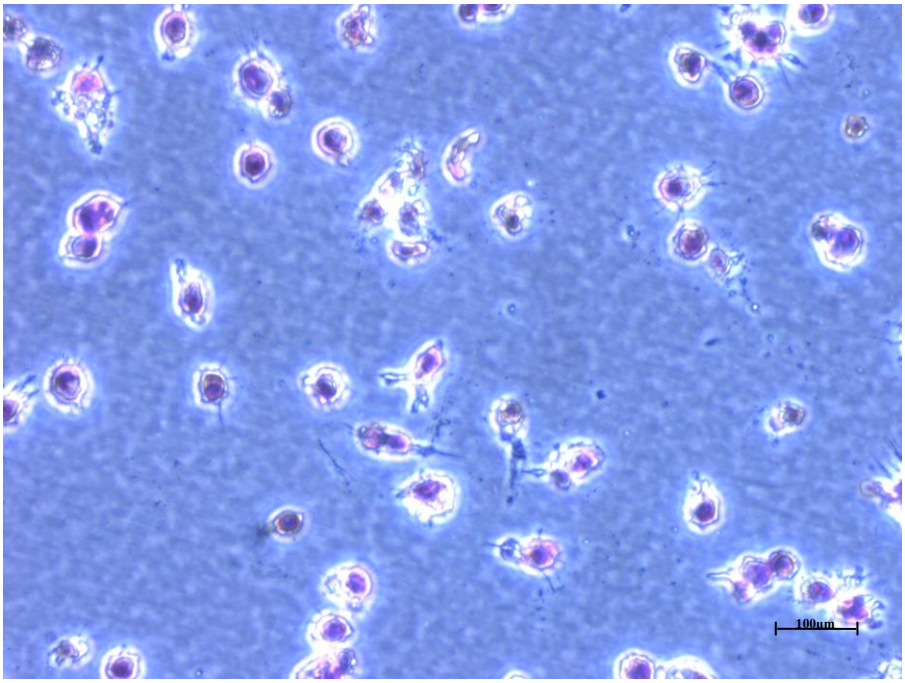


Figure 3.1.5: Morphological differences between alveolar and peritoneal macrophages.

AMs and PMs recovered from cryopreservation were selected by an adherence step (overnight). Both cell populations show the usual stellate, adherent morphology of macrophages. The AMs ***(A)*** *were larger in size, more granular in appearance and developed more pseudopodia compared to PMs* ***(B).*** *Figures show light microcopy images. Scale bar = 100µm.*

A



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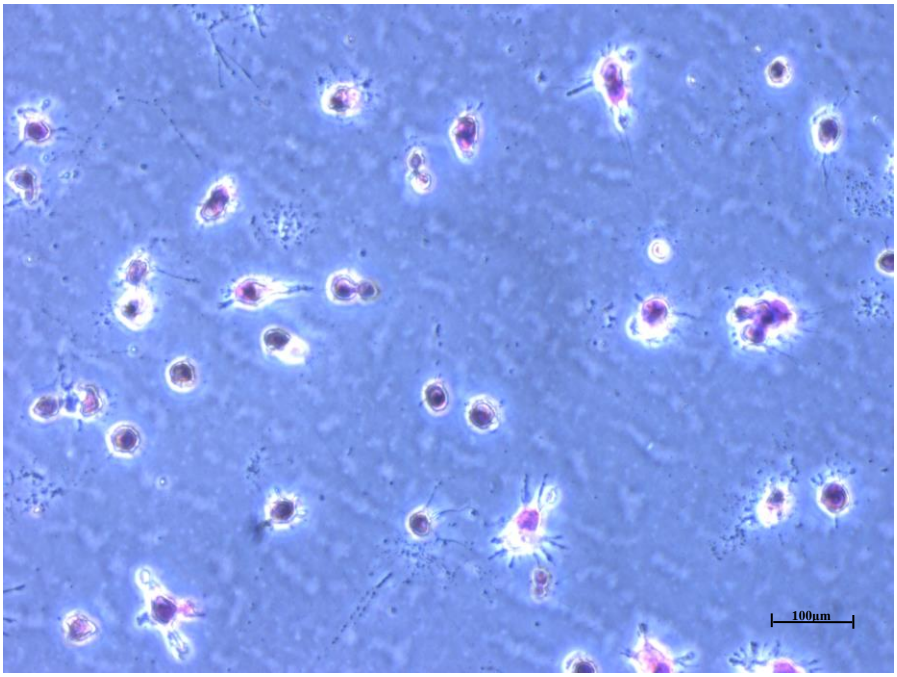


Figure 3.1.6: Leishman stain on cell culture plate of AMs (A) and PMs (B) for investigation of adherent cell differentiation (>90% were macrophages).

Figures show confocal microscopy images. Scale bar = 100µm.

3.1.5 The response of equine macrophages to LPS is dependent on the type of serum used in the culture medium

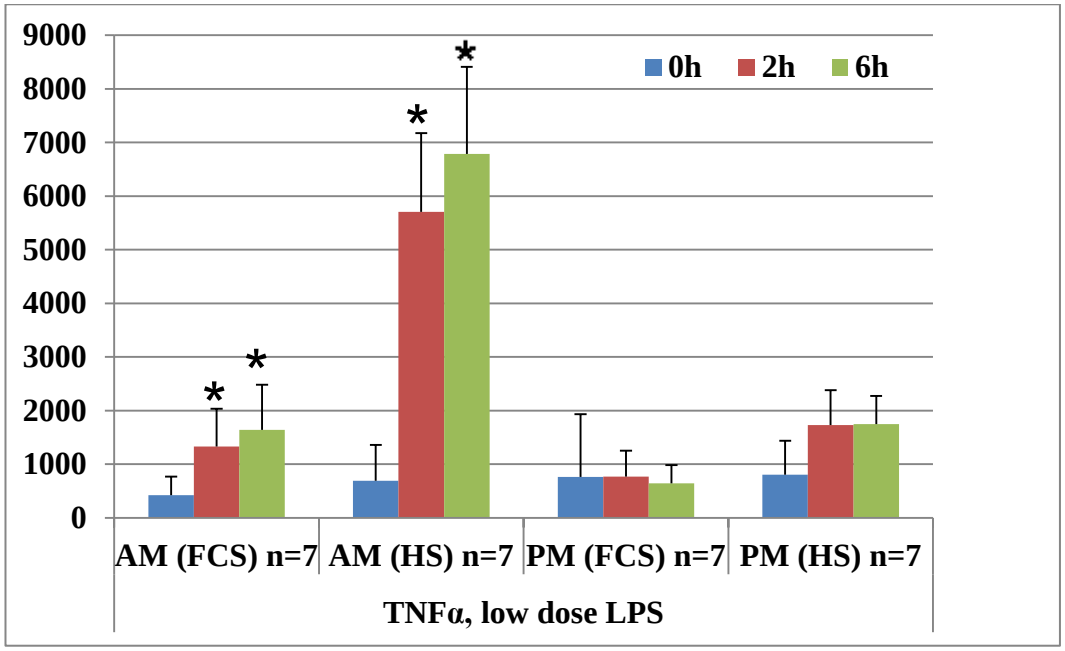
Serum is an essential component of cell culture media, and may be obtained from the autologous adult source (e.g. horse serum for equine studies) or from heterologous sources, commonly fetal bovine serum, newborn calf serum, adult human serum or pig serum (Werners et al., 2004, Okano et al., 2006, Fogelman et al., 1980, Pleau et al., 1977, Tenang and McCaldin, 1988). All sources of serum contain many basic nutrients, vitamins, growth factors, hormones and binding proteins, such as LBP and complement components, that augment the ability of the cells to respond to stimuli, opsonise particles and participate directly in various biological processes (Jochems et al., 2002, Zweigner et al., 2006). In particular, LBP is present in serum of healthy mammals and it enhances LPS binding to soluble, as well as cell membrane-bound CD14 of phagocytes in order to initiate the intracellular signaling pathway (Zweigner et al., 2006, Martin et al., 1992). Following stimulation with LPS, LBP is recruited to transfer LPS to CD14 on the surface of macrophages/monocytes, a process followed by formation of a complex with TLR4 and MD2, initiation of NFKB signaling pathway, activation and cytokine production (Heumann et al., 1992, Steinemann et al., 1994, Miyake, 2004). LBP enhances the cell sensitivity to LPS, permitting the detection of, and assessment of the response to, very low LPS concentrations (pg/mL) (Steinemann et al., 1994, Van Amersfoort et al., 2003). Serum, as a blood product, also contains complement, which might result in complement mediated cell lysis, an effect inhibited by heat inactivation. Heat-inactivated FCS has been a common serum of choice for cell culture experiments of murine and human cells (Heumann et al., 1992, Steinemann et al., 1994, Puddu et al., 1997). One key molecule that is apparently absent from FCS, but normally present in adult serum, is CSF1. This factor is commonly assayed in the presence of FCS in many studies (Gow et al., 2012, Gow et al., 2013).

Previous studies on horse macrophages have examined the importance of serum in monocyte responsiveness. Okano *et al* (2006) showed that LPS-induced pro-coagulant activity by equine mononuclear cells was greater in the presence of FCS,

compared to serum free medium (Okano et al., 2006). Another study found that pooled commercial equine serum or autologous equine serum produced more consistent results from cellular assays of equine monocyte cultures compared to heat-inactivated autologous equine serum or heat-inactivated FCS (Figueiredo et al., 2008). Heat-inactivated autologous equine serum was found to have relatively low LBP activity, whereas FCS directly stimulated equine macrophages (Figueiredo et al., 2008). Moreover, commercial horse and autologous horse serum showed more consistent results between horses compared to FCS (Figueiredo et al., 2008).

The relative merits of different sera were initially examined by comparing the response of freshly-isolated equine AMs and PMs to LPS in the presence of heat-inactivated FCS versus heat-inactivated HS. The cells were plated at a concentration of 10^6 cells/mL and left to rest overnight in heat-inactivated FCS or HS. The following day, medium was refreshed and cells were stimulated with two different concentrations of LPS. There was no apparent morphological difference between the cells incubated in the two different sera. The supernatant concentration of TNFα was measured prior to and 2h and 6h following stimulation of cells with low (10ng/mL) or high (100ng/mL) LPS concentrations **(Figure 3.1.7)**. LPS-induced TNFα production by AMs was detected by 2h when cells were incubated with either serum, and was significantly higher in the presence of HS at this and the later time point (6h). The low concentration of LPS was almost maximally stimulatory to AMs. In contrast to AMs, PMs released TNFα constitutively at similar levels when cultured in the presence of both HS and FCS, and there was no further induction in the presence of LPS, even at the higher concentration of 100ng/mL **(Figure 3.1.7)**. The aim of the present study was to use appropriate conditions for studying equine AM biology *in vitro*. Since equine AMs showed a higher ability to respond to LPS while incubated with HS compared to FCS. Therefore, heat inactivated HS was used for the rest of the experiments performed in this thesis.

A



B

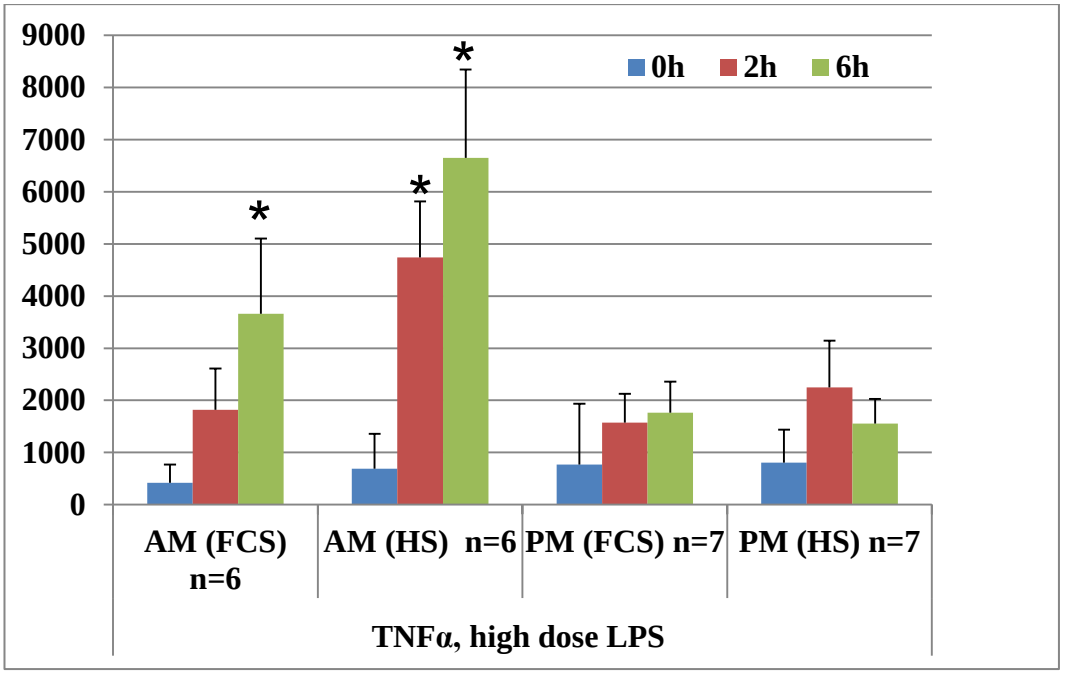


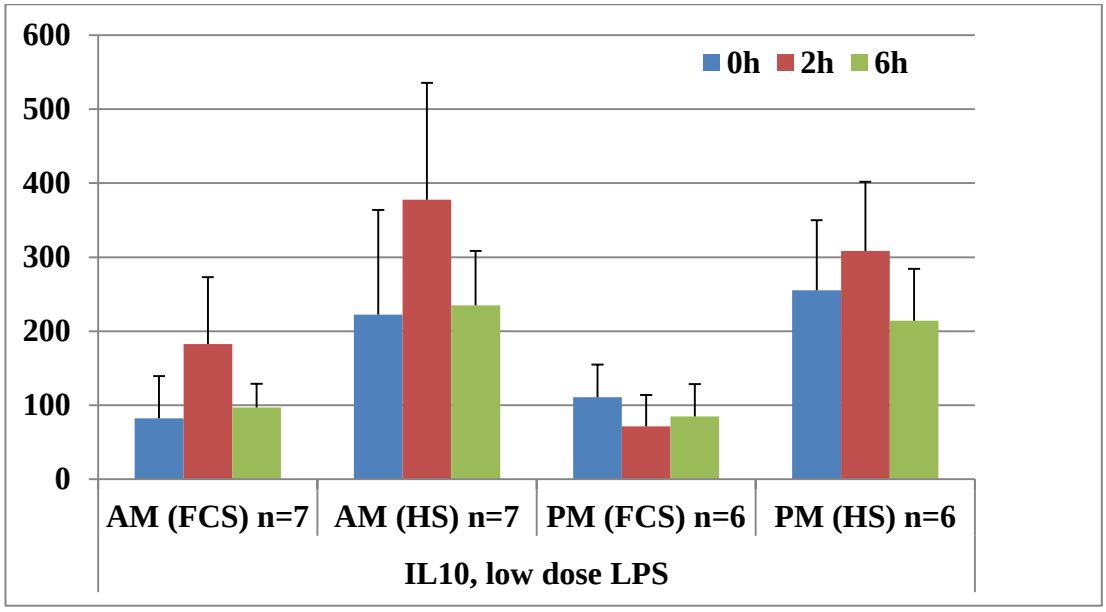
Figure 3.1.7: TNFα (pg/mL) production from freshly harvested AMs and PMs in response to low (10ng/mL) (A) and high (100ng/mL) LPS doses (B) and different heat inactivated sera (HS and FCS).

Alveolar and peritoneal cells were seeded at 10^6 cells/mL and left to rest overnight. The next day non-adherent cells were washed away and cell medium was refreshed with FCS or HS. Fresh AMs and PMs were stimulated with LPS for 0h, 2h and 6h. The y axis shows the TNFα (pg/mL) production from

freshly harvested AMs and PMs in response to low (10ng/mL) ***(A)*** *and high (100ng/mL)* ***(B)*** *LPS doses. Results are the mean of a minimum of 6 experiments +/- SEM (*p<0.05 versus control 0h).*

Macrophages stimulated with LPS also produce IL10, a well-recognized anti-inflammatory cytokine, which exerts feedback inhibition of the signaling cascade (Bogdan et al., 1992). IL10 can be produced by Th2 cells and B cells, as well as macrophages and monocytes in mice (Mosmann and Moore, 1991, Ogarra et al., 1990). Laan *et al* (2005) previously reported that LPS treated horse AMs produce mRNA for IL10 (Laan et al., 2005), and human IL10 significantly reduced the production of inflammatory mediators (Hawkins et al., 1998). It was considered that one possible explanation for the apparent refractoriness of PMs to LPS could be IL10 production. However, IL10 levels remained almost undetectable in both AMs and PMs using a commercially available ELISA and were not increased by LPS in either FCS or HS **(Figure 3.1.8)**. In all ELISAs performed throughout the study, a standard curve was generated for each assay according to the manufacturers instruction **(Section 2.4).** Results were only considered valid if the correlation coefficient (r^2) value was >0.9. The detection limit of both the IL10 and TNFα assays was 500pg/ml.

A



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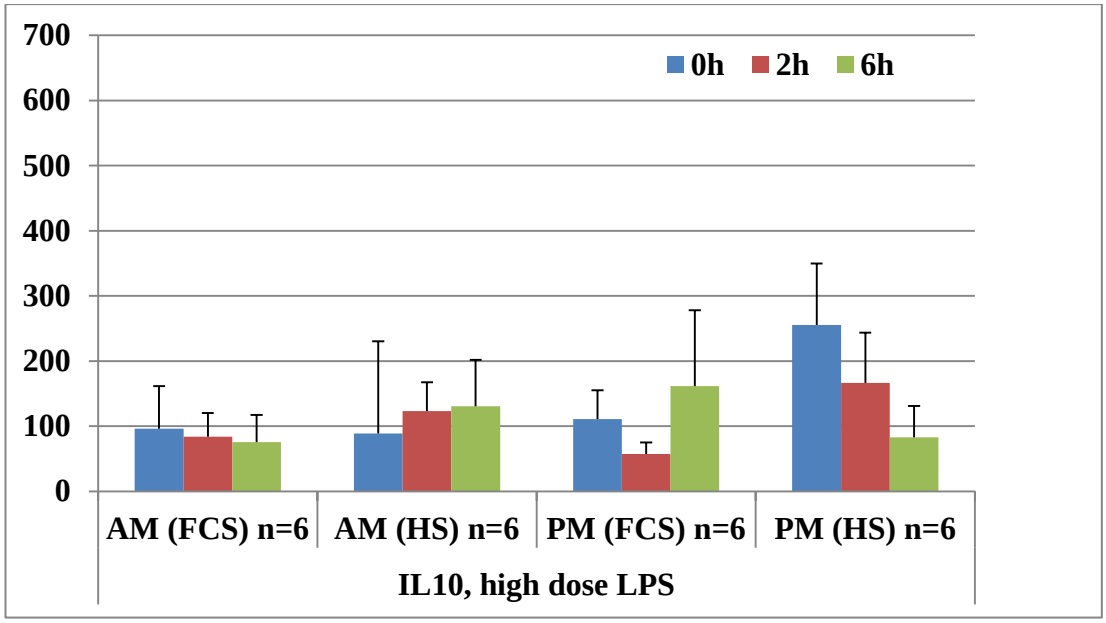


Figure 3.1.8: AM and PM production of IL10 (pg/mL) in response to low (10ng/mL) and high (100ng/mL) LPS doses when incubated in different heat inactivated sera (HS and FCS).

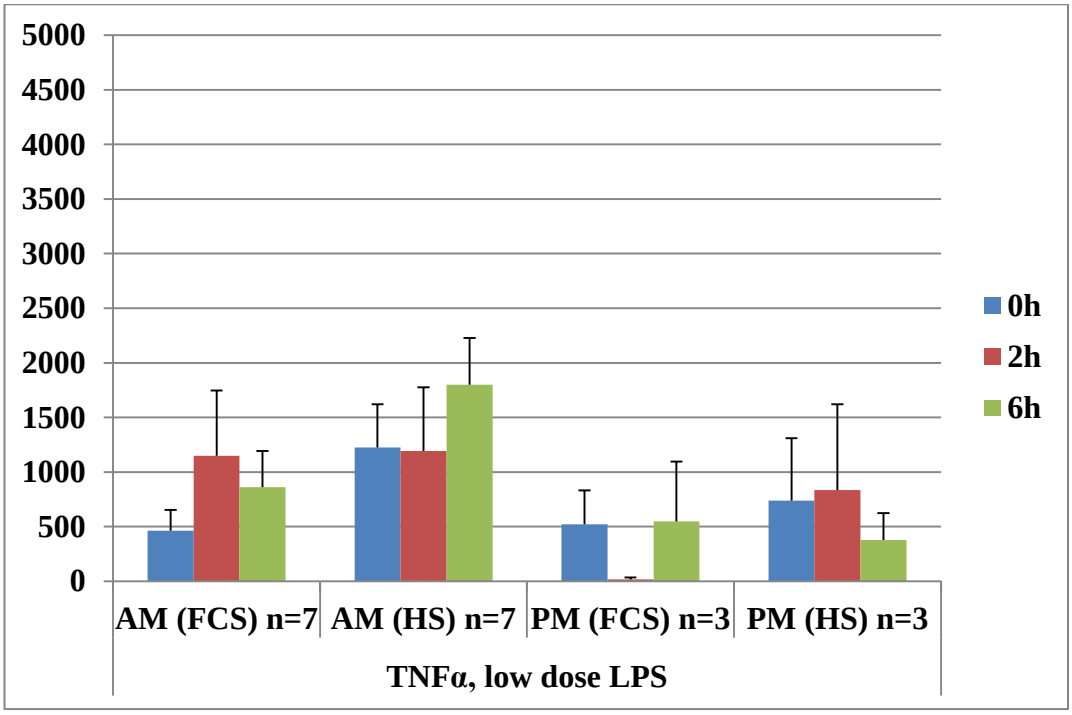
Alveolar and peritoneal cells were seeded at 10^6 cells/mL and left to rest overnight. The next day non-adherent cells were washed away and cell medium was refreshed with FCS or HS. Fresh AMs and PMs were stimulated with low (10ng/mL) ***(A)*** *and high dose of LPS (100ng/mL)* ***(B)*** *for 0h, 2h and 6h. IL10 production was measured by ELISA on cell culture supernatants. Results are the mean of a minimum of 6 biological replicates +/- SEM. The y axis shows the IL10 production in pg/ml.*

3.1.6 Cryopreserved AMs and PMs, following LPS stimulation, show a similar pattern of cytokine response as the freshly harvested cells

Compared to fresh cells, AMs recovered following cryopreservation exhibited a reduction in the absolute magnitude of LPS-induced TNFα release. However, there was no change in the time course of the LPS response, nor the relatively greater response in HS compared to FCS **(Figure 3.1.9)**. Similarly, the PMs recovered from cryopreservation showed the same lack of response to LPS, as observed with fresh cells. The cryopreserved cells also failed to release detectable IL10 **(Figure 3.1.10)**.

Because IL10 is an anti-inflammatory cytokine, its production may be delayed, compared to TNFα, following exposure to a stimulus (Liese et al., 2001). To test this hypothesis, the time course of stimulation of AMs was extended to 24h. At this time point, there was no further elevation in TNFα production by the AMs, no detectable TNFα production by PMs and the IL10 production by AMs and PMs remained less than 250pg/mL **(Figure 3.1.11)**.

A



B

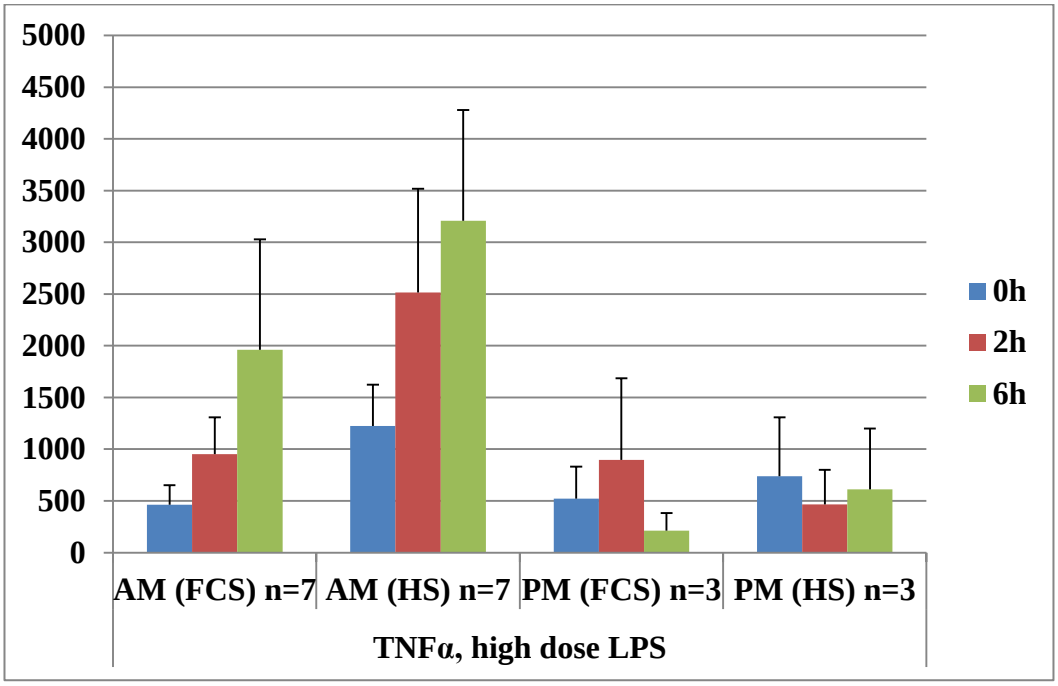
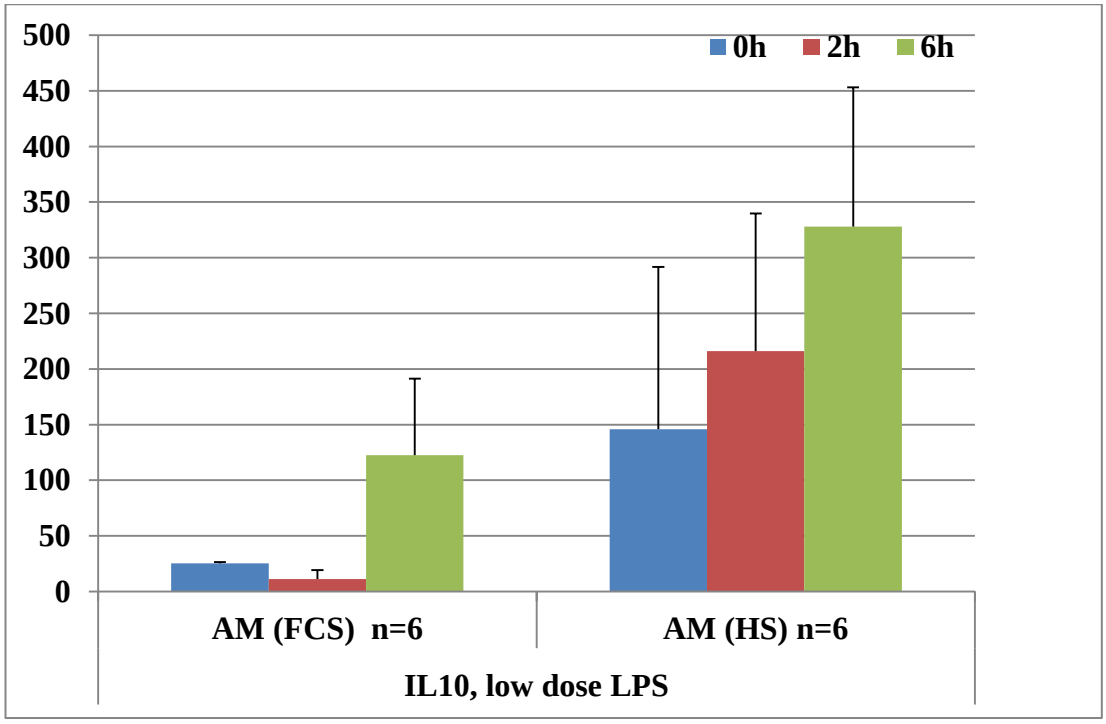


Figure 3.1.9: TNFα (pg/mL) production by cryopreserved AMs and PMs in response to low (10ng/mL) and high (100ng/mL) dose LPS in different sera (heat inactivated HS and FCS).

Cells were cultured as previously described in ***Figure 3.1.8****. The y axis shows the TNFα production in pg/ml.Results are the mean of a minimum three experiments +/- SEM.*

A



B

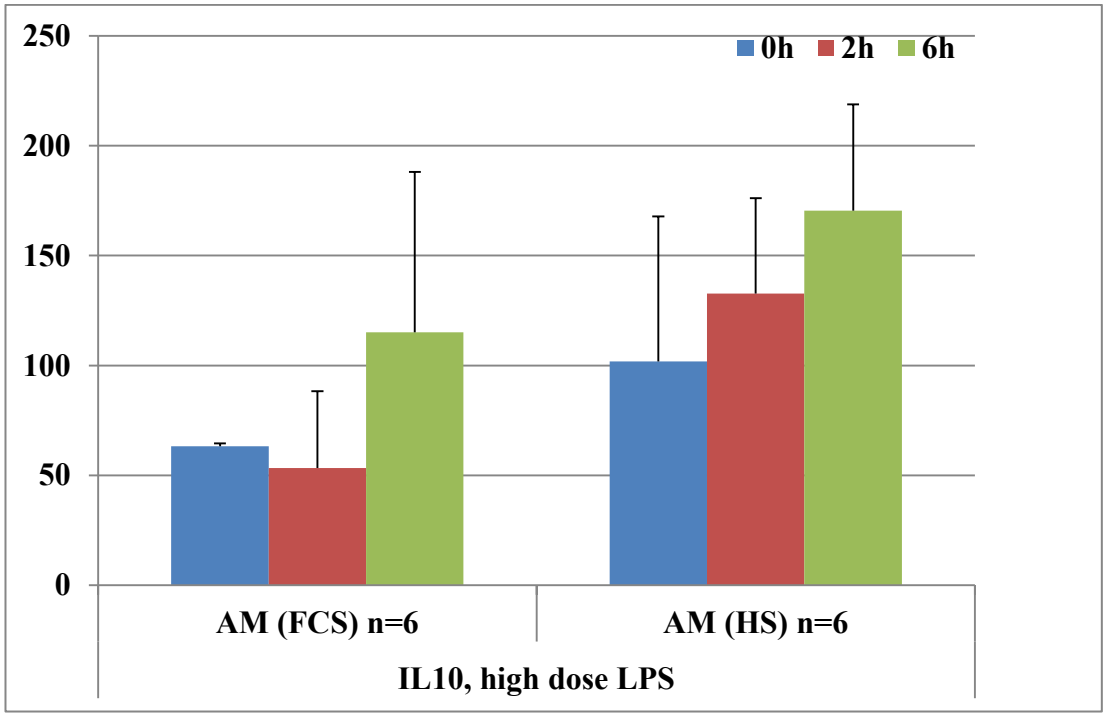


Figure 3.1.10: IL10 (pg/mL) production by cryopreserved AMs in response to stimulation with low (10ng/mL) and high (100ng/mL) dose LPS, incubated in different sera (heat inactivated HS and FCS).

The y axis shows the IL10 production in pg/ml. Results are the mean of 6 experiments +/- SEM.

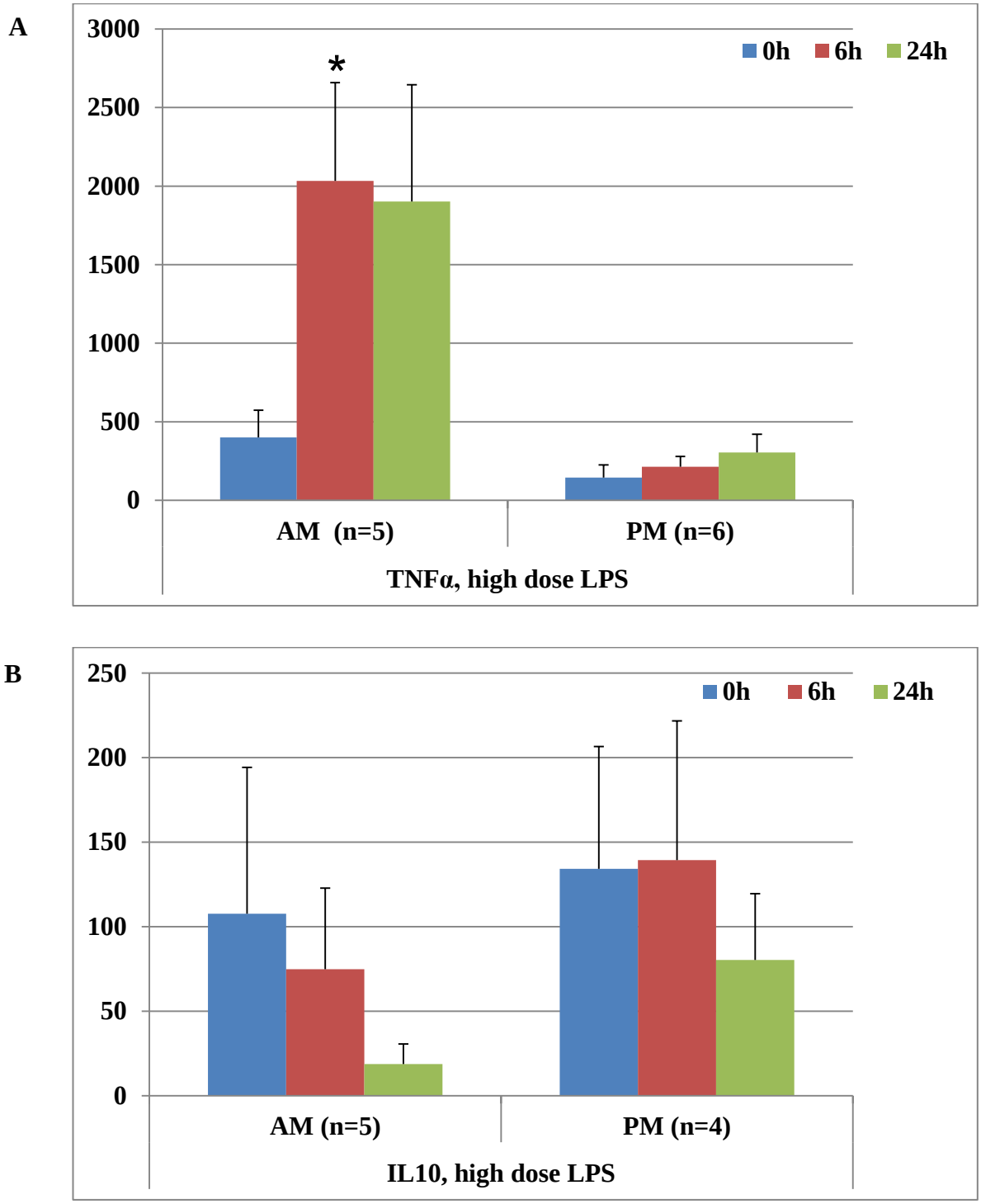


Figure 3.1.11: TNFα and IL10 production (pg/mL) at 24h.

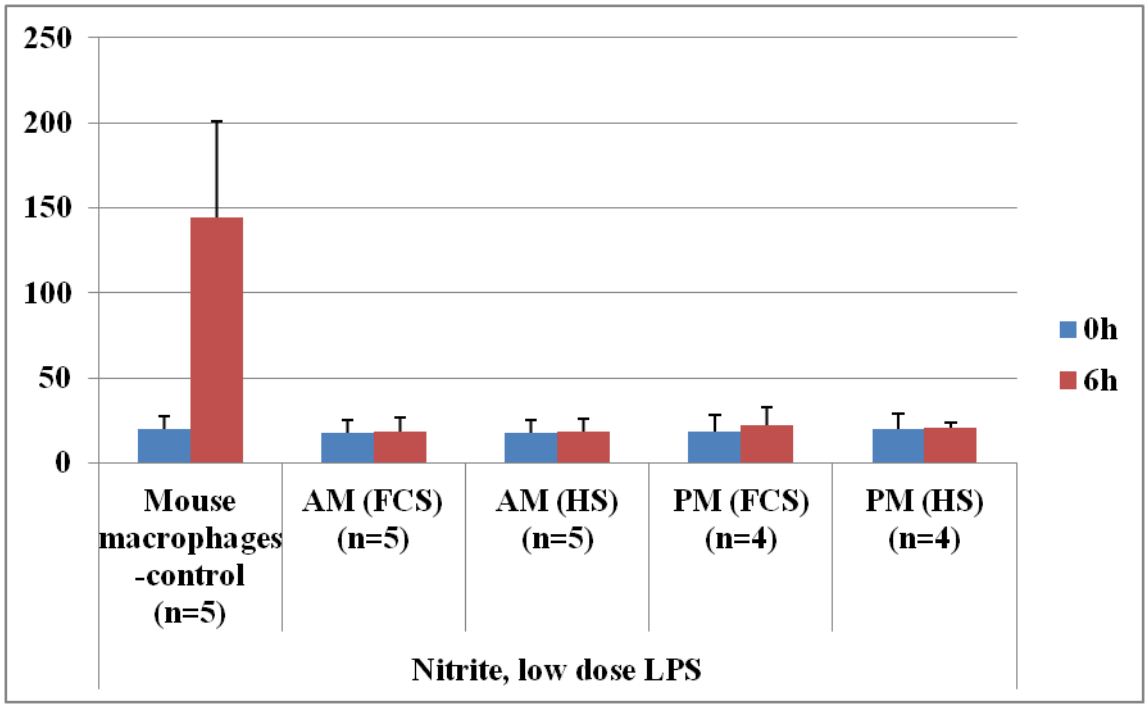
TNFα (pg/mL) ***(A)*** *and IL10 (pg/mL)* ***(B)*** *production at 24h by AMs and PMs following stimulation with high dose (100ng/mL) LPS. Cells were incubated in medium supplemented with heat inactivated HS. The y axis shows the TNFα* ***(A)*** *and IL10* ***(B)*** *production in pg/ml. Results are the mean of a minimum of four experiments +/- SEM.*

3.1.7 In contrast to LPS treated mouse macrophages, equine AMs and PMs did not produce NO

Nitric oxide (NO) has been attributed many functions in host defense based largely on the results of studies performed in rodents. LPS stimulation of mouse macrophages increases arginine uptake and inducible nitric oxide synthase (iNOS) activity and stimulates production of NO (Ito et al., 2005, Takacs et al., 2012). Neither pig nor human macrophages produce NO in response to LPS (Kapetanovic et al., 2012, Schneemann et al., 1993). There are some conflicting reports as to whether horse AMs produce NO (Hammond et al., 1999a, Johnson et al., 1997). Hammond *et al* (1999) reported that stimulation of equine lung macrophages with LPS induced iNOS and nitric oxide production. Conversely, Johnson *et al* (1997) failed to identify NO production by equine macrophages. To further address this question, nitrite production by fresh and cryopreserved AMs and PMs was measured following stimulation with LPS (10ng/mL and 100ng/mL) for 6h. The mouse macrophage cell line RAW 274.1, known to produce NO in response to LPS, was used as a positive control. Although, the LPS-stimulated RAW274.1 cells produced abundant nitrite, no nitrite was detected in the supernatant of LPS-stimulated equine AMs or PMs either before **(Figure 3.1.12)** or after cryopreservation **(Figure 3.1.13)**.

The mouse iNOS promoter is not conserved in the pig or human genome (Zhang et al., 1996, Kapetanovic et al., 2012). In order to investigate whether the differences in nitrite production between the species were controlled at a transcriptional level, iNOS promoter sequence alignment (2000bp in front of the start codon) was performed in the three species: human, mouse and horse (retrieved from the Ensembl database; *www.ensembl.org*). Using the Geneious software, the conservation of the sequences between species using a Pustell DNA matrix, was investigated (http://www.geneious.com). Human and horse promoters are well conserved as shown in **Figure 3.1.14.A**, whereas there is little alignment of the promoter sequences between mouse/horse **(Figure 3.1.14.B)** and mouse/human **(Figure 3.1.14.C)**.

A



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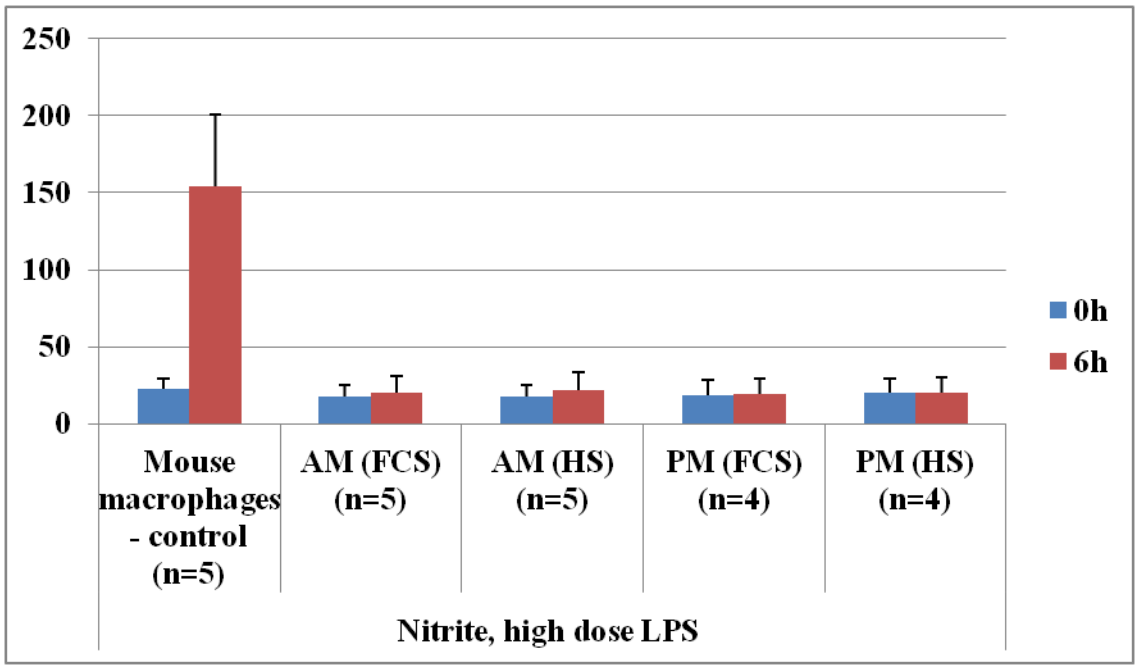
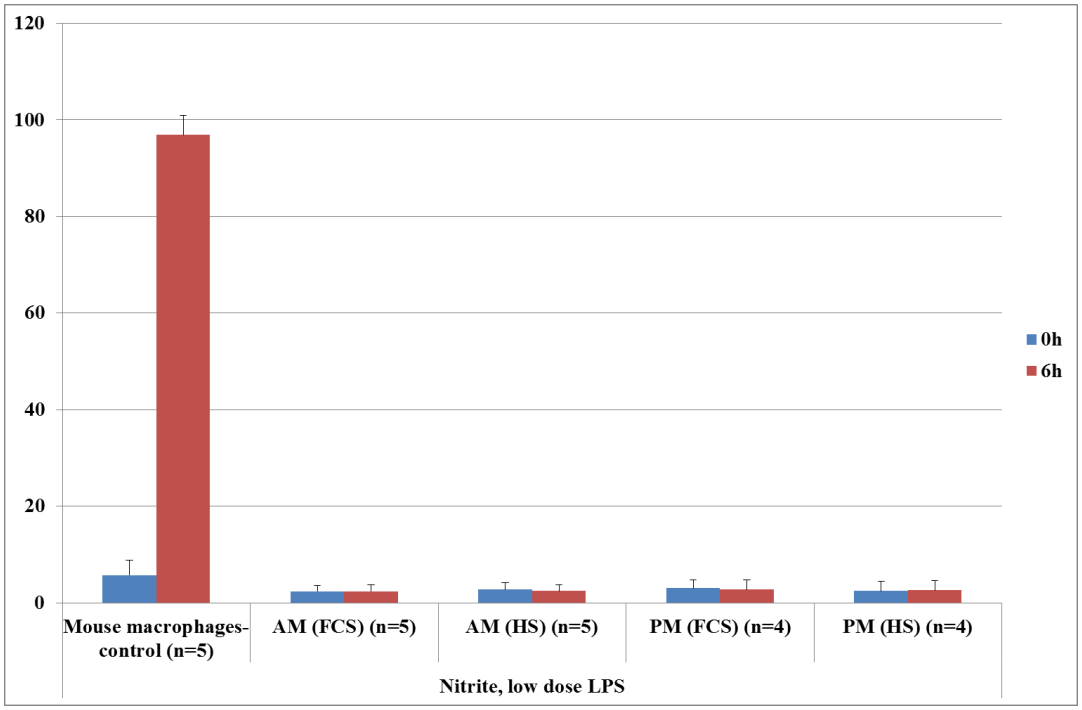


Figure 3.1.12: Nitrite production was induced in murine but not in fresh equine AMs and PMs.

Mouse RAW274.1 macrophages and fresh horse macrophages were treated similarly with low (10ng/mL; ***A)*** *and high (100ng/mL;* ***B)*** *dose LPS and nitrite (µM/mL) was measured in the supernatant using the Griess reagent as described in Materials and Methods Section. Results are the mean of a minimum of three experiments +/-SEM.*

A



B

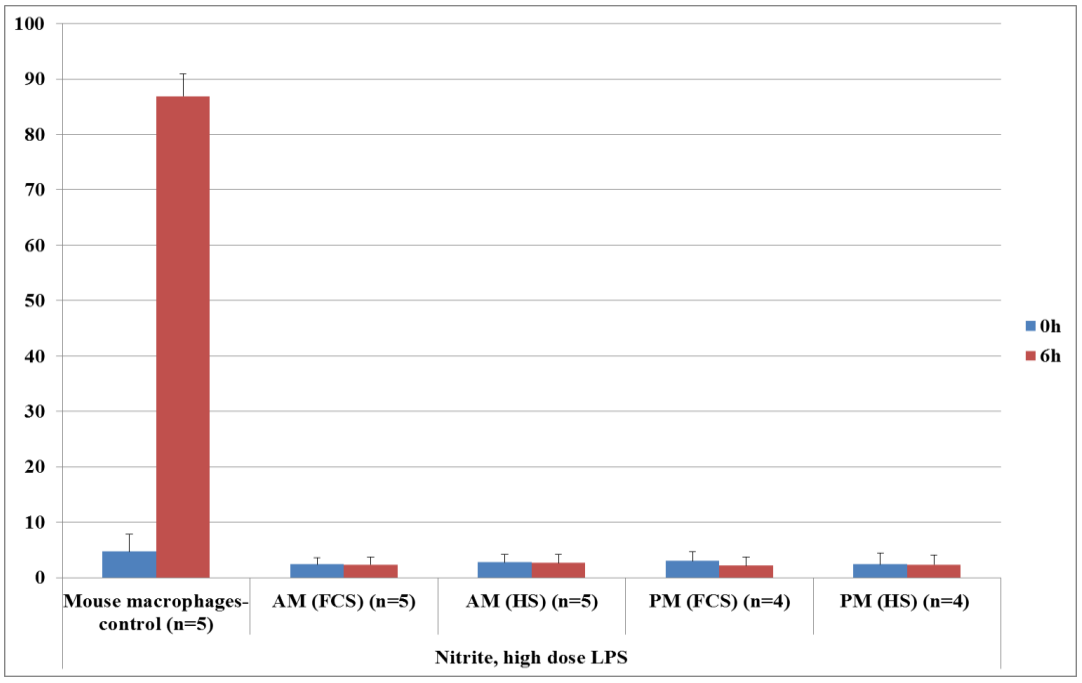


Figure 3.1.13: Nitrite was induced in mouse but not in cryopreserved equine macrophages.

*Mouse and cryopreserved horse macrophages were treated similarly with low (10ng/mL; **A)** and high (100ng/mL; **B)** dose LPS and nitrite (µM/mL) was measured in the supernatant using the Griess reaction. Results are the mean of a minimum of four experiments +/- SEM.*

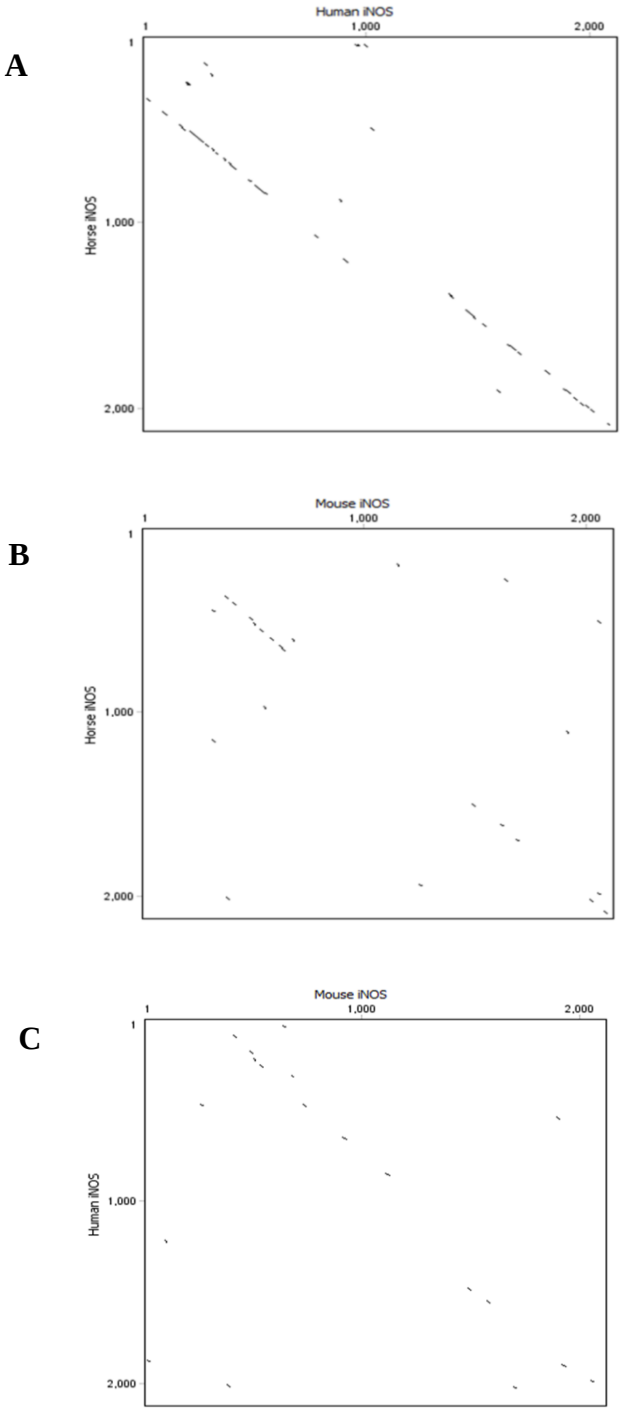


Figure 3.1.14: Dot plots of iNOs promoter sequences of human, horse and mouse.

Promoter sequence of iNOS was compared between horse, mouse and human using Pustell graph. Sequence up to 2000bp 5' of the starting codon of the three species were aligned pairwise; horse versus human ***(A)****, horse vs mouse* ***(B)*** *and human vs mouse* ***(C)****.*

3.1.8 Equine AMs and blood monocytes show endotoxin tolerance

As discussed in the introduction **(Section 1.4.1)**, a natural feedback mechanism, known as endotoxin tolerance, is recruited in cases of continuous exposure to LPS in order to provide some protection to the host against excessive inflammation and tissue damage. The role of macrophages/monocytes in this mechanism is critical and has been widely described in humans and mice (Hotchkiss and Karl, 2003, Karin et al., 2006, Biswas and Lopez-Collazo, 2009).

To model the endotoxin tolerance in AMs, they were seeded at 10^6 cells/mL and left to rest overnight. The next day nonadherent cells were washed away and the cell medium was refreshed (0h timepoint). AMs were stimulated with 100ng/mL of LPS and left to incubate for 24h. The following day, after the supernatant was collected for TNFα measurement, cells were re-stimulated with the same dose of LPS and incubated for another 24h **(Figure 3.1.15)**. Controls were either not stimulated at all, or stimulated only on the first or second day. Supernatants were collected after the 1st (24h time point) and 2nd (48h timepoint) day and kept frozen for subsequent TNFα measurement by ELISA. The same experiment was performed with horse monocytes.

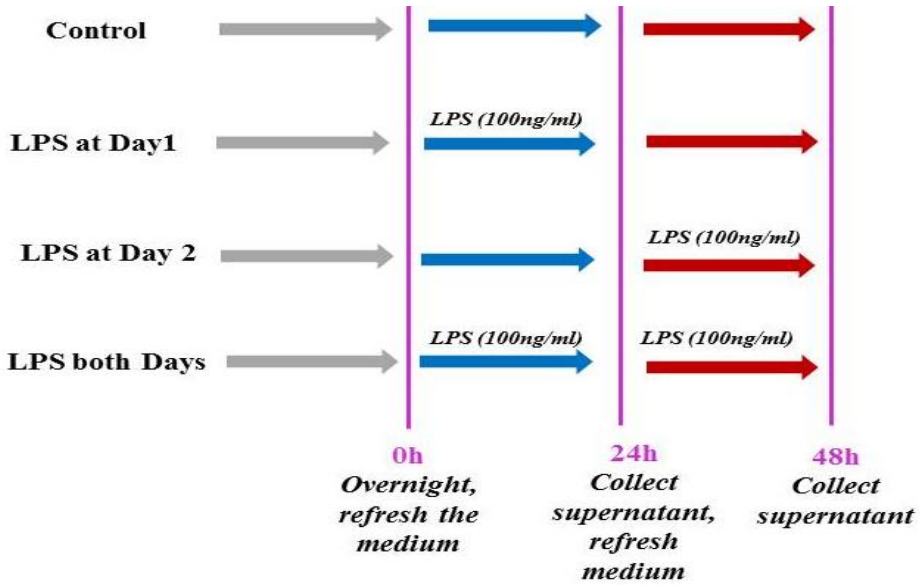
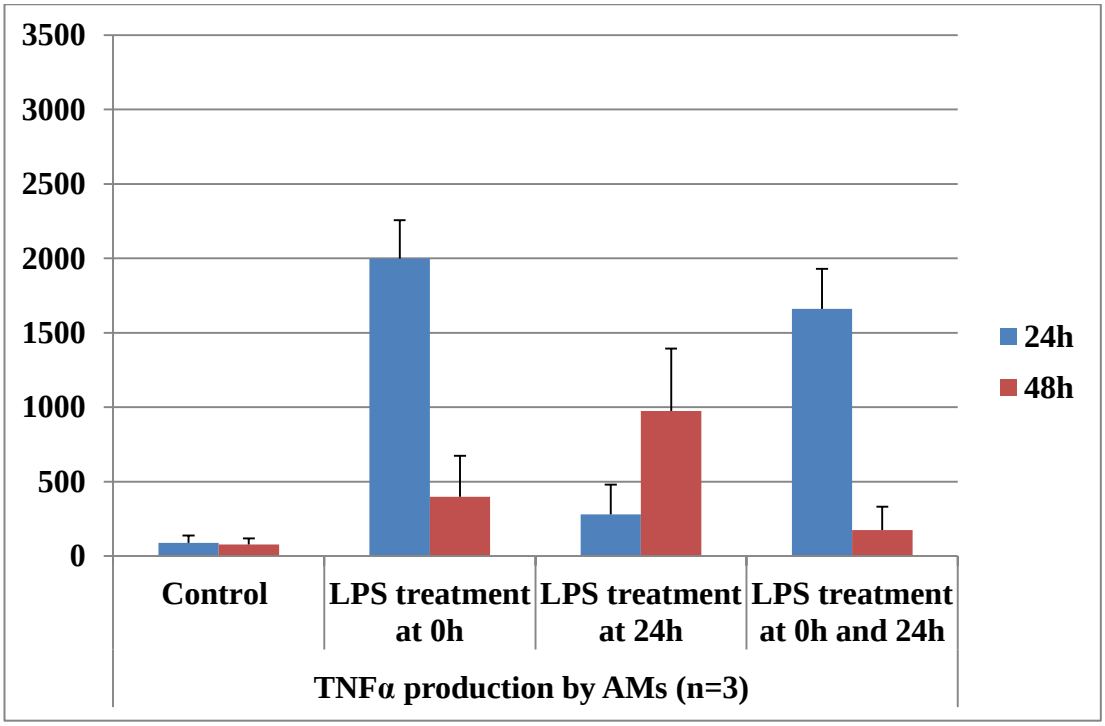


Figure 3.1.15: Experimental design of endotoxin tolerance experiment on AMs and monocytes.

The results of the sequential treatment are shown in **Figure 3.1.16**. AMs that were incubated in medium only (controls) did not produce high levels of TNFα throughout the experiment. In AMs that were treated with LPS on day 1 only, as expected, TNFα concentration was increased in the supernatant collected 24h following LPS stimulation. However, following a further 24h incubation in medium only, TNFα levels in the supernatant subsequently declined. In AMs which were cultured in medium only for the first 24h, then stimulated with LPS at this time point (24h), the TNFα concentration of the supernatant was elevated although to a lesser extent than in fresh cells, probably due to some loss of cells during cell culture. Finally, further (day 2) LPS treatment of AMs that were treated with LPS on day 1 did not restimulate TNFα production in the second 24 hours. In fact, TNFα production was lower compared to AMs given medium alone on day 2. Essentially the same pattern was seen in blood monocytes **(Figure 3.1.16-B)**. Slight production of TNFα was observed when the cells were incubated with cell culture medium only, for 24h. This level was considered insignificant (less than 300pg/mL for AMs and 500pg/mL for monocytes) and might have been due to some interaction of the cells.

Consistent with the previous experiments, AMs did not produce detectable IL10 under any of the conditions studied **(Figure 3.1.17)**. By contrast monocytes did produce a significant amount of IL10 when stimulated with LPS, thereby providing a positive equine control for the assay and supporting the conclusion that the previous failure to detect high IL10 was an AM specific defect. The pattern of regulation of IL10 was different from TNFα. Inducibility increased after 24 h in culture, and did not decline to the same extent following re-addition of LPS.

A



B

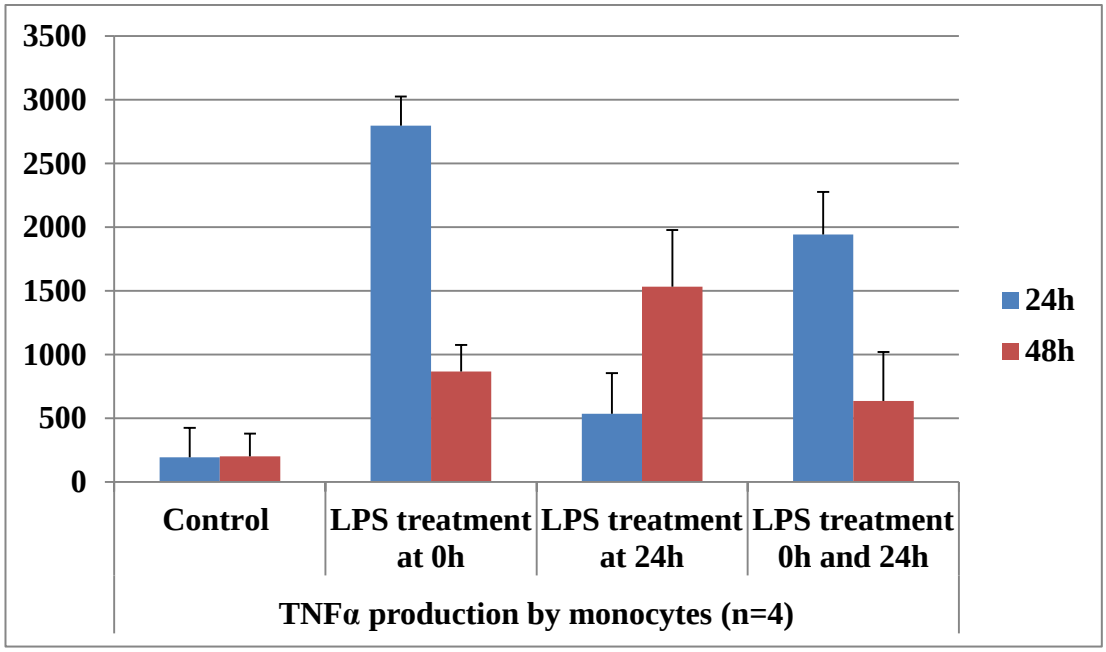


Figure 3.1.16: TNFα (pg/mL) production by LPS tolerant AMs and blood monocytes.

*AMs **(A)** and blood monocytes **(B)** were treated with the same LPS dose (100ng/mL) on two consecutive days (0h and 24h) as shown in **Figure 3.1.15**. Cell culture supernatant was collected at 24h and 48h and TNFα (pg/mL) production was measured by ELISA. Control cells were cultured in medium only, at all time points. Results are the mean of a minimum of three experiments +/- SEM. The y axis shows the TNFα production in pg/ml.*

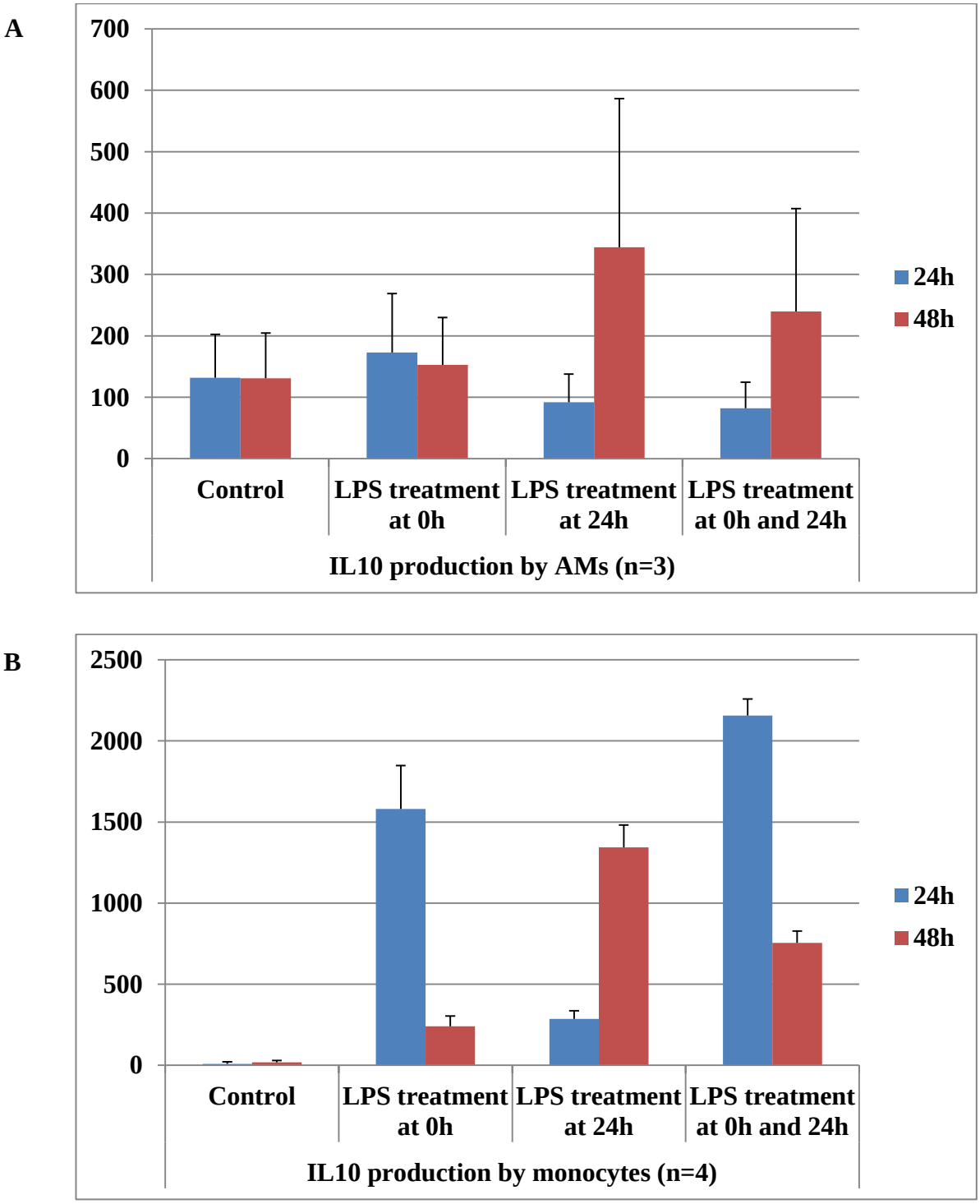


Figure 3.1.17: IL10 production (pg/mL) by LPS tolerant AMs and blood monocytes.

*AMs **(A)** and blood monocytes **(B)** were treated with the same LPS dose (100ng/mL) on two consecutive days. Cell culture supernatant was collected and IL10 (pg/mL) production was tested by ELISA. The y axis shows the IL10 production in pg/ml.Results are the mean of a minimum of three experiments +/- SEM.*

3.1.9 rhCSF1 and high concentration of HS induced differentiation of horse PBMCs to macrophages

CSF1 is required for the differentiation and development of macrophages in embryonic and adult life (Chitu and Stanley, 2006, Gow et al., 2010, Hamilton and Achuthan, 2013). To establish an *in vitro* macrophage culture for the horse, monocytes were cultured for seven days in rhCSF1. As shown in **Figure 3.1.18**, by the end of the culture period, the majority of cells was larger, spread on the substratum and resembled mature macrophages. Differentiation of macrophages derived from several species such as human (Chinetti et al., 1998) and mice (Wiktor-Jedrzejczak et al., 1982) can also be achieved by incubating monocytes in cell culture medium without CSF1 supplementation. In these experiments monocytes differentiated into macrophages only in the presence of only serum and cell culture medium. Likewise, in the current study, equine monocytes grown in medium supplemented with 30% HS differentiated to macrophages **(Figure 3.1.19)**.

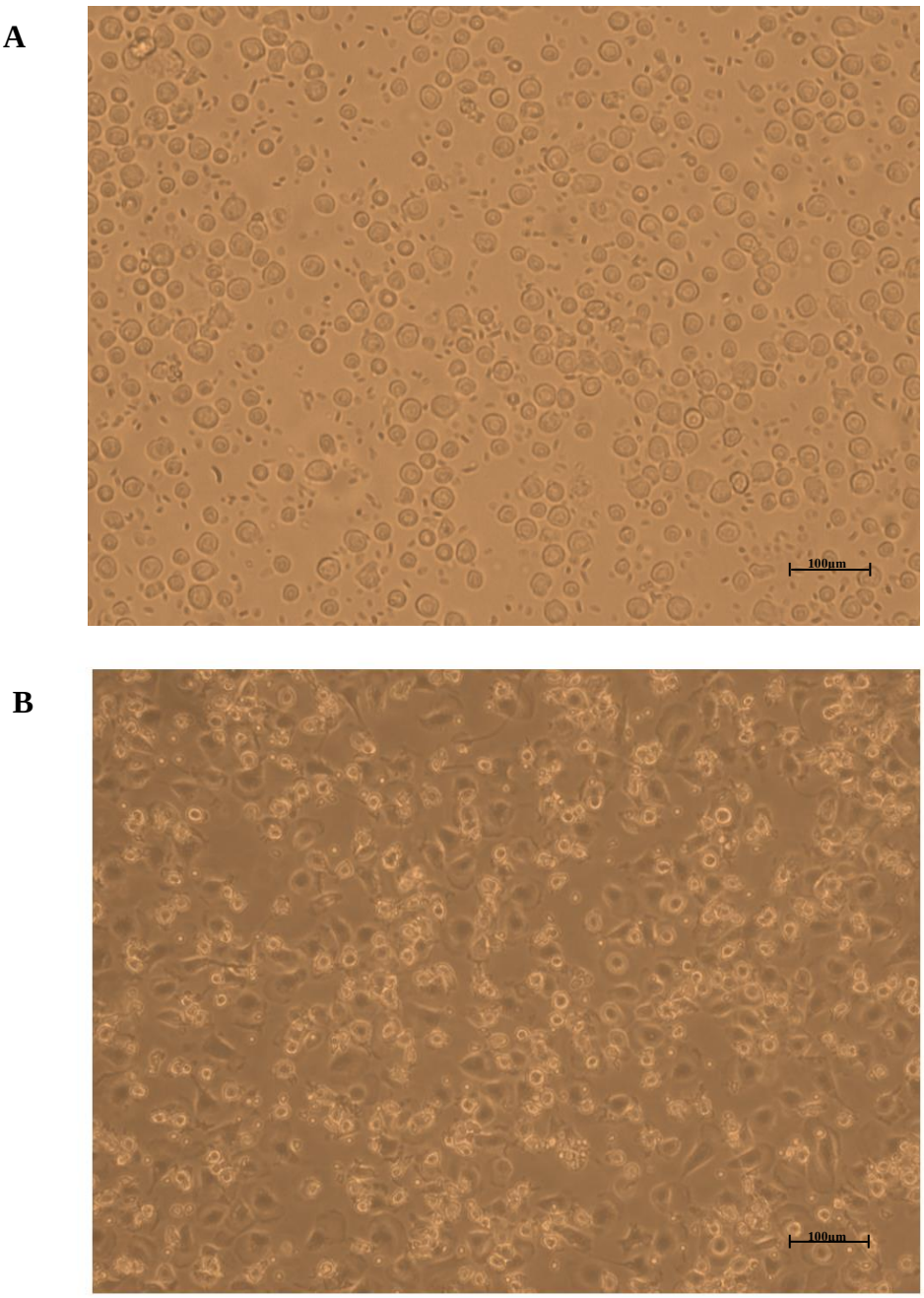
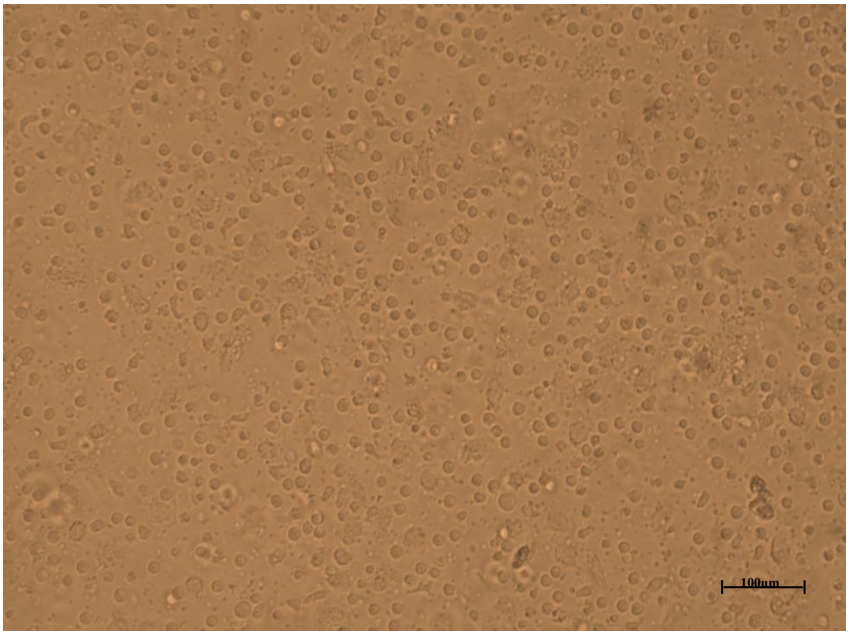


Figure 3.1.18: Equine PBMC-derived macrophages.

PBMCs were stimulated with rhCSF1 at day 1 ***(A)****. Monocyte-derived macrophages at day six* ***(B)****. Figures show light microcopy images. Scale bar = 100µm.*

A



B

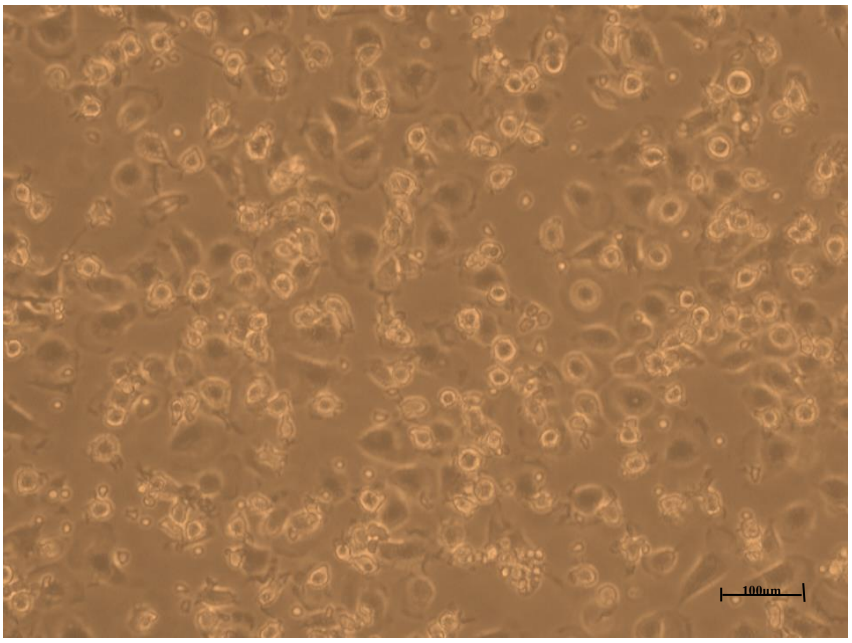


Figure 3.1.19: Equine PBMC-derived macrophages.

PBMCs were stimulated with 30% heat-inactivated HS at Day 1 ***(A)****. Monocyte derived macrophages at day six* ***(B)****. Figures show light microcopy images. Scale bar = 100µm.*

3.1.10 CD14, CD163 and TLR4 are present on equine blood monocytes

Blood monocytes have been more widely studied than other types of horse monocytes/macrophages, because they are relatively easy to collect (Watson et al., 1990, Grunig et al., 1991). However, their characterisation has not been complete because of the lack of commercially available horse specific reagents. Most studies have relied on the crossreactivity of several anti-human or anti-mouse antibodies against equine proteins (Steinbach et al., 2005, Ibrahim et al., 2007). The cell membrane proteins CD14 and CD163 are expressed by equine monocytes (Steinbach et al., 2005, Kabithe et al., 2010). Kabithe *et al* (2010) have produced an equine specific CD14 antibody, which was used in the current study (Kabithe et al., 2010). However, TLR4 expression on equine blood monocytes has not been tested using flow cytometry.

To test the available reagents, PBMCs were seeded at 10^6 cells/mL and left to rest overnight. The next day nonadherent cells were washed away and the adherent cells were detached using a cell scraper and stained for flow cytometry. Size (FS) and granularity (SS) were used to distinguish different cell populations and their gating as shown in **Figure 3.1.20-A**. **Figure 3.1.20.B-D,** demonstrate that CD14, TLR4 and CD163 could be detected by flow cytometry on equine blood monocytes. The fluorescence intensity is shown in **Figure 3.1.21**. This provided an appropriate comparative dataset for results presented in **Section 3.2** using the equine AMs and PMs. A continuum of CD163 expression was detected on the cells, although it was not fully established whether this reflected 2 distinct sub-populations, as described with porcine monocytes (Fairbairn et al., 2013b). Fairbairn and colleagues (2013) demonstrated two pig monocyte populations $CD14^{high}$ $CD163^{low}$ and $CD14^{low}$ $CD163^{high}$ that more closely resembled human monocyte populations than those of mice, as discussed in the introduction **(Section 1.2)**. Wether this is the case in horse needs to be further investigated.

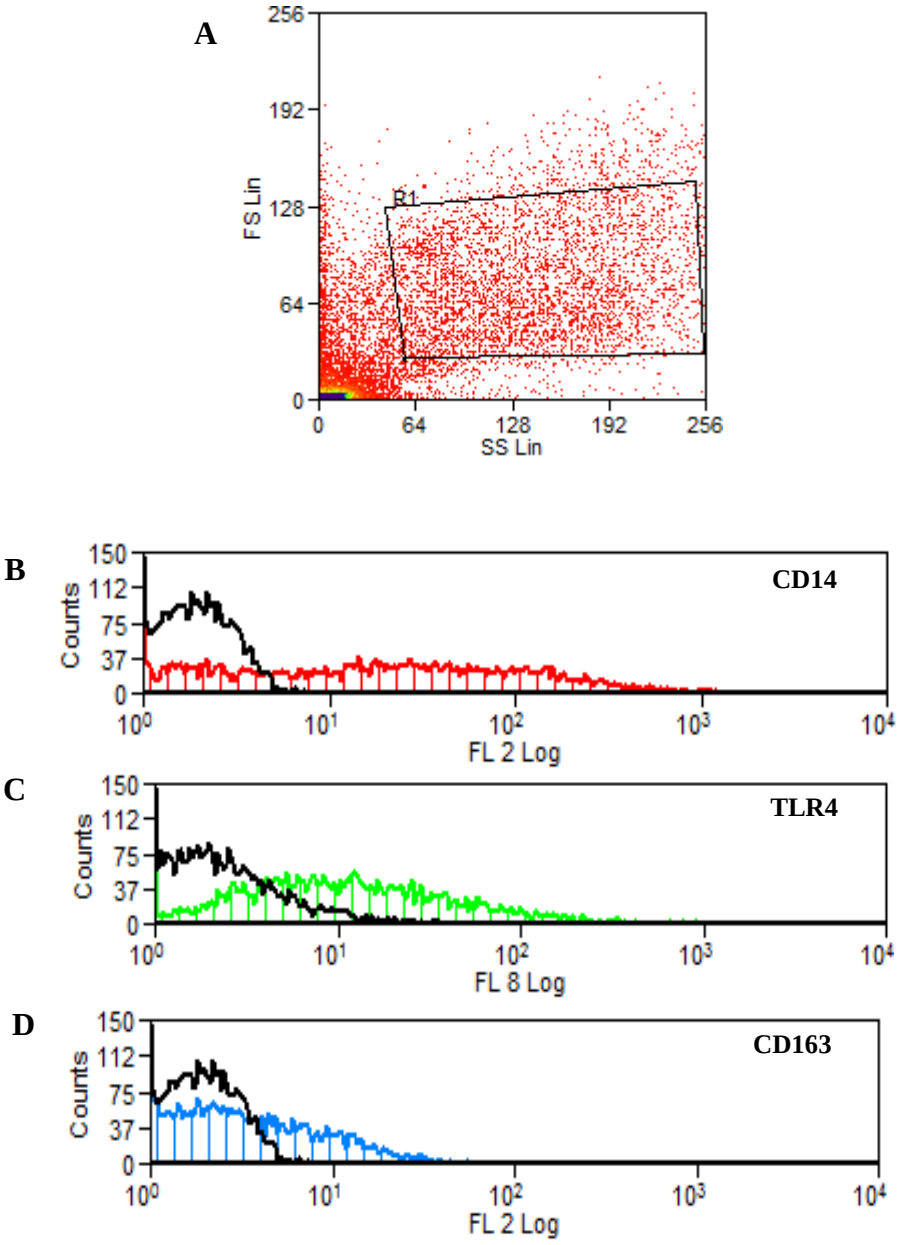


Figure 3.1.20: CD14, CD163 and TLR4 expression on equine monocytes.

*After isolation PBMCs were seeded at 10^6 cells/mL and left to rest overnight. The next day non-adherent cells were washed away and cells were stained for flow cytometry. Size (FS) and granularity (SS) were used for cell distinction and gating (A). Cells were stained for CD14, TLR4 and CD163. Results are shown in figures **B-D** and respectively. Isotype controls are represented by a non-hatched black curve and targeting antibody is represented by a coloured hatched curve. Figures are representative of one out of a minimum of 3 experiments.*

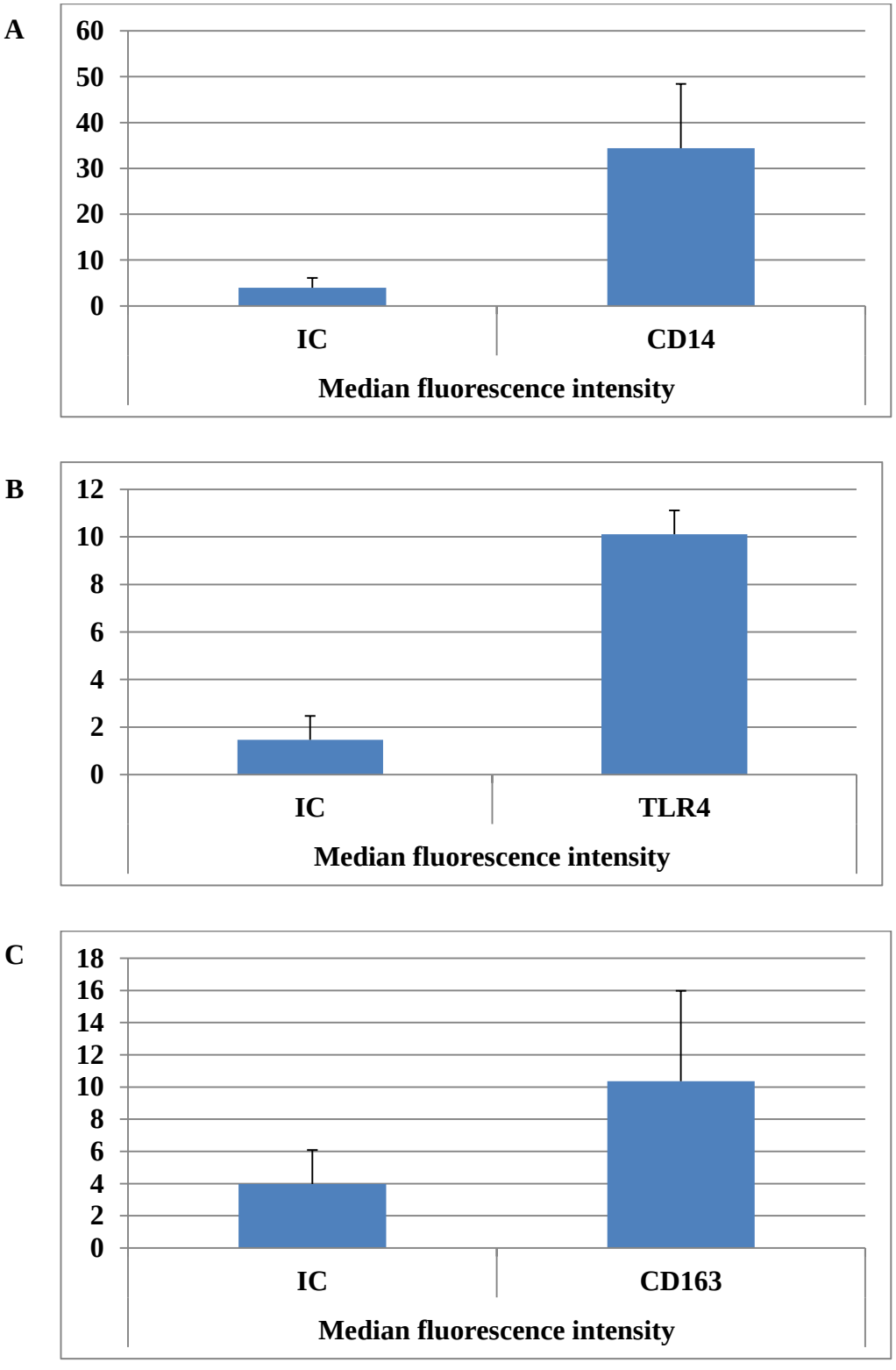


Figure 3.1.21: Median fluorescence intensity of CD14 (A), TLR4 (B) and CD163 (C) and appropriate isotype control (IC) expression on equine blood monocytes.

Results are the mean of a minimum of 3 experiments +/- SEM.

3.2 Equine alveolar macrophages differ in their function and phenotype from peritoneal macrophages

As discussed in detail in the introduction **(Section 1.5.2)**, there is considerable evidence from other species that AMs adapt to their environment in a unique way and are distinct from macrophages derived from other locations, a finding thought to reflect the distinctive microenvironment of this cell and its "breath-by-breath" exposure to ambient air containing a variety of potential allergenic and pro-inflammatory components, as well as its different origin (Guth et al., 2009, Salez et al., 2000, Nibbering et al., 1987, Landsman and Jung, 2007). Therefore, it was considered essential to further characterize the basic function and phenotype of the equine AM by making specific comparisons with other tissue derived macrophages, such as PMs.

3.2.1 AMs, but not PMs, respond to different inflammatory stimuli

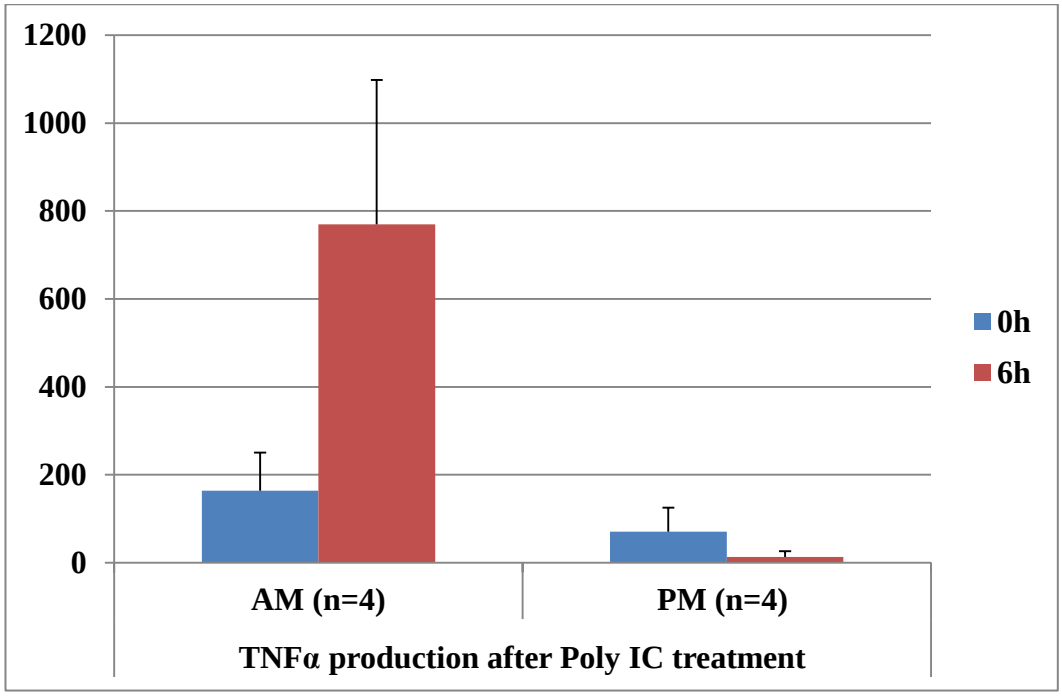
To investigate the relative responsiveness of AMs and PMs to LPS, cells were plated at a concentration of 10^6 cells/ml and left to rest overnight in heat-inactivated HS, in the optimal conditions determined in **Section 3.1.5**. As previously shown **(Section 3.1.5)**, AMs, but not PMs, responded to LPS treatment. To determine whether the lack of responsiveness of PMs was specific to the TLR4 agonist, both AMs and PMs were stimulated with other agonists containing alternative ligands. Thus, after overnight culture, medium was refreshed and cells were stimulated with either heat-killed bacteria (*Salmonella typhimurium*; 10^6cfu/ml, 10^7cfu/ml and 10^8cfu/ml) or Poly IC (20µg/ml), a TLR3 ligand. The doses of the stimulants used were similar with those tested in previous monocyte/macrophage experiments in different species including horse (Kapetanovic et al., 2012, Waldschmidt et al., 2013, Yusof et al., 1993).

Salmonella typhimurium is a serovar of the *Salmonella enterica* subsp. *enterica*, which is a member of the genus *Salmonella* (gram negative bacteria) and is a major

cause of food borne salmonellosis (Prescott et al., 1996, Voetsch et al., 2004). *Salmonella typhimurium* is also an important equine pathogen, resulting in enteritis and associated endotoxaemia, a clinical status which, in horses, carries an uncertain prognosis (Peet et al., 1980, Durando et al., 1994, Werners et al., 2005). The heat-killed *Salmonella typhimurium* used in this study contains a mixture of TLR4 (LPS), TLR2 (peptidoglycan), TLR9 (CpG) and TLR5 (flagelin) ligands (Prescott et al., 1996, Kwon et al., 2011).

AMs produced TNFα in response to both heat-killed *Salmonella typhimurium* **(Figure 3.2.1)** and Poly IC **(Figure 3.2.2)**. As previously noted following LPS stimulation, **(Section 3.1.5)**, IL10 levels remained almost undetectable in both AMs and PMs at all timepoints.

A



B

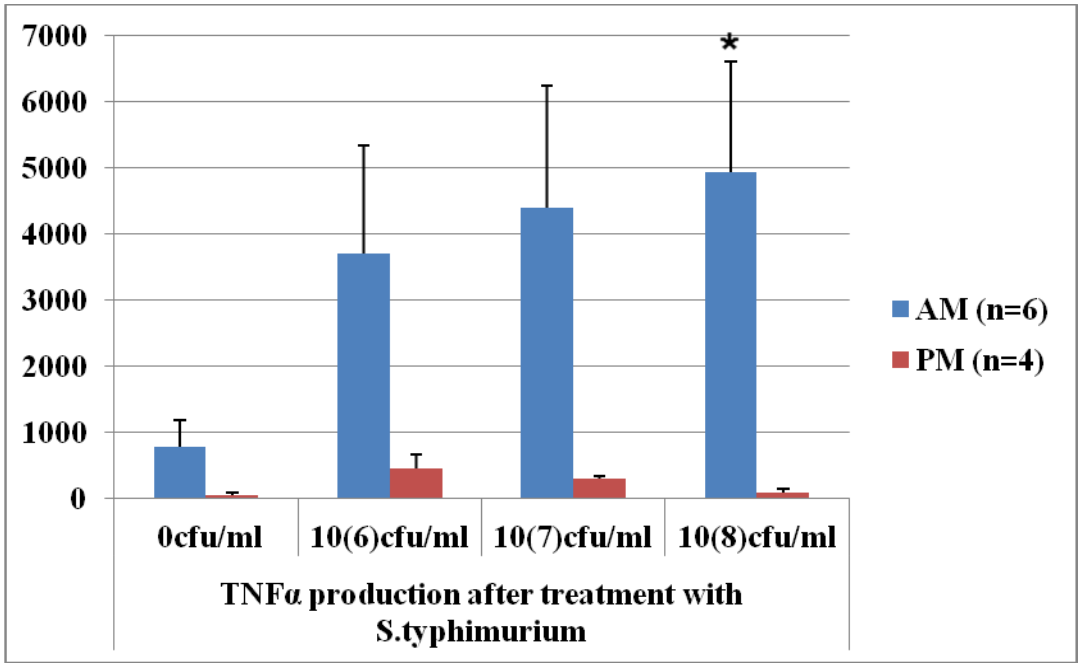
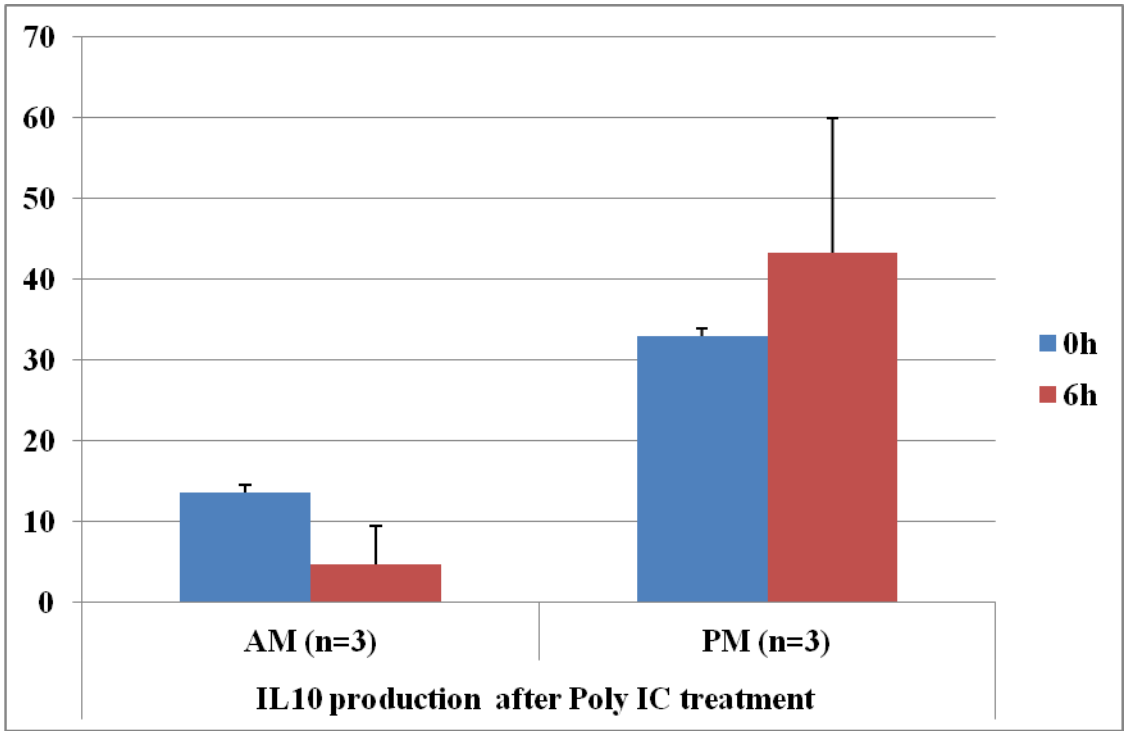


Figure 3.2.1: TNFα (pg/ml) production of AMs and PMs in response to inflammatory stimuli. *Alveolar and peritoneal cells were seeded at 10^6 cells/ml and left to rest overnight. The next day non-adherent cells were washed away and cell media was refreshed with HS. AMs and PMs were stimulated with 2µg/ml of Poly IC for 6h* ***(A)*** *and with 3 doses of Salmonella typhimurium (10^6, 10^7 and 10^8 cfu/ml)* ***(B)****. The y axis shpws the TNFα (pg/ml) production of AMs and PMs in response to inflammatory stimuli. Results are the mean of a minimum of four experiments performed with samples of different horses +/- SEM (*p<0.05 versus control 0h).*

A



B

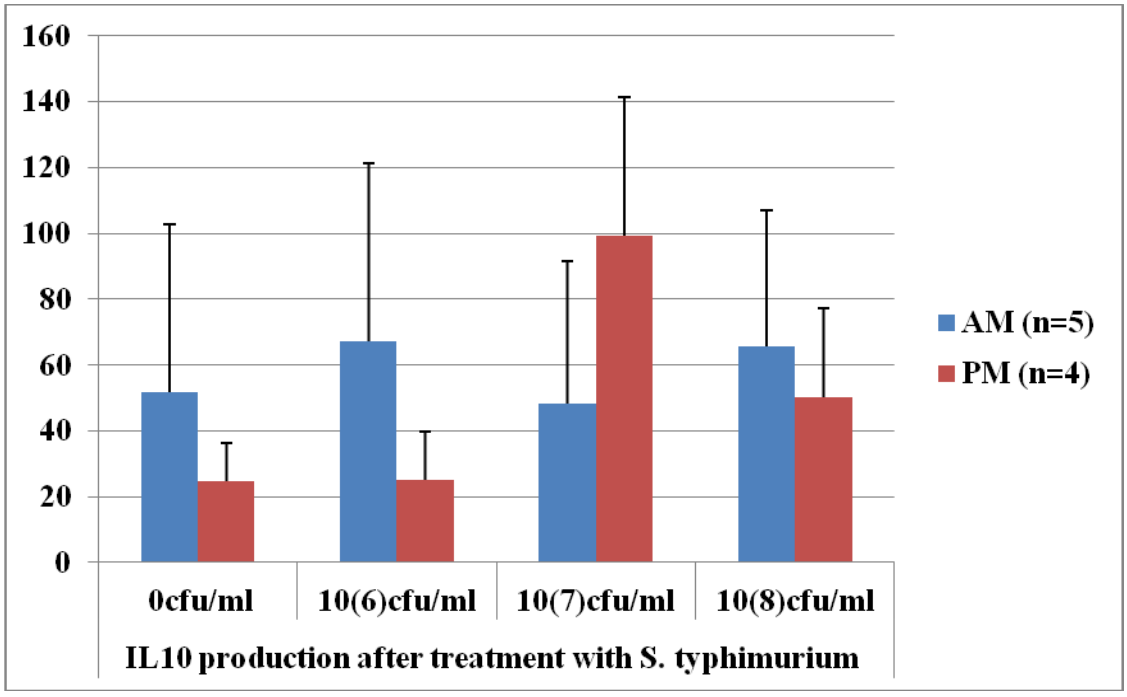


Figure 3.2.2: IL10 (pg/ml) production of AMs and PMs in response to inflammatory stimuli.

*Cells were treated as in **Figure 3.2.1**. AMs and PMs stimulated with 2µg/ml of Poly IC for 6h **(A)** and with three doses of Salmonella typhimurium (10^6, 10^7 and 10^8 cfu/ml) **(B)**. The y axis shpws the IL10 (pg/ml) production of AMs and PMs in response to inflammatory stimuli. Results are the mean of a minimum of 3 experiments +/- SEM (*p<0.05 versus control 0h).*

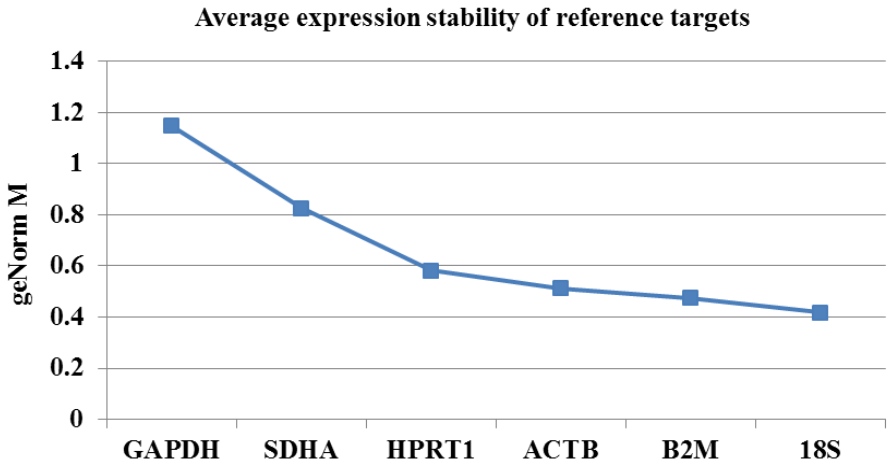
3.2.2 Identification of suitable reference genes for RT qPCR assay in AMs

RT qPCR is a widely used assay for reliable and quick quantification of gene expression under specific conditions (Arya et al., 2005, Peirson and Butler, 2007). The relative gene expression in different treated groups is quantified and normalised to a reference gene (Arya et al., 2005). The reference gene should remain stable under different treatment protocols or disease conditions (Schmittgen and Zakrajsek, 2000, Cappelli et al., 2008). Since the expression of reference genes is tissue dependent, optimal reference gene selection for the tissue studied is essential for reliable RT qPCR results (Zhang et al., 2009).

Reference gene selection was based on widely published housekeeping genes used in several horse studies and was performed on four LPS treated and four untreated AM populations derived from systemically healthy horses. *GAPDH* and *ACTB* were used in many studies on BALF derived cells of different species including horse (Laan et al., 2006, Perkins et al., 2008, Riihimaki et al., 2008, Reyner et al., 2009, Beekman et al., 2011, Hughes et al., 2011) and other genes such as *HPRT1*, *SDHA*, *18S*, *B2M*, *28S* have been investigated in other equine tissue cells (Cappelli et al., 2008, Zhang et al., 2009). In order to select the most appropriate reference gene for the equine macrophage-based studies, the M-value, describing the variation of a gene compared to all other candidate genes, was used. The gene with the lowest M-value is considered the most stable (Vandesompele et al., 2002). Six candidate reference genes were ranked based on their M-value, as assessed using qBase^{plus} software. All reference genes had M-values less than 1.5, the threshold below which genes are considered to be stably expressed. *18S* rRNA expression was found to be the most stable compared to *B2M*, *ACTB*, *HPRT1* and *SDHA* **(Figure 3.2.3.A)**. Interestingly, the commonly used *GAPDH* was the least stable gene of the group. If more than one housekeeping gene is required, the measurement of the GeNorm V value of the genes under review is considered a suitable way to determine the optimum number required for an expression study (Vandesompele et al., 2002). The pairwise variation between samples is decreased by the inclusion of more reference genes (Vandesompele et al., 2002), thus indicating the number of genes required to achieve an arbitrarily selected

threshold of reference gene stability (Vandesompele et al., 2002). A V value of 0.15 is considered a suitable threshold (Perez et al., 2008). In the current protocol, the optimal combination of reference genes that could be used had a V value of 0.15 and included the following genes; *18S, B2M* and *ACTB* **(Figure 3.2.3.B)**.

A



B

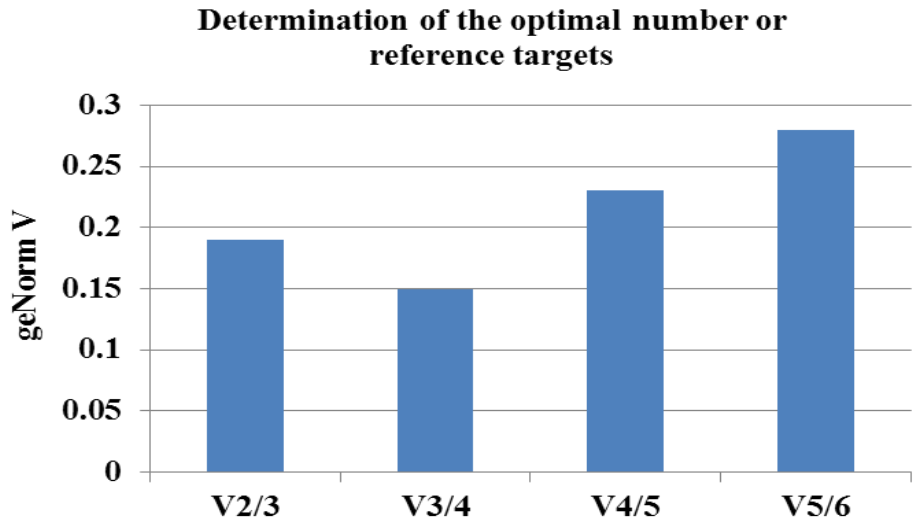


Figure 3.2.3: Gene expression stability of candidate reference genes.

*qBase^{plus} software was used for the ranking of the six candidate reference genes based on their M-value **(A)**. The lower the M-value, the higher the stability of the gene. qBase^{plus} software calculates the optimal number of reference genes that should be used in order to calculate the normalization factor **(B)**. The lower the V value, the better the gene combination.*

Results were consistent with the findings of Zhang *et al* (2009) who found no significant differences in expression of *18S* and *ACTB* between equine colon, small intestine, heart, spleen, kidney, liver, lung and lymph nodes (Zhang et al., 2009). *18S,* selected in previous studies on equine pulmonary-derived and blood mononuclear cells (Ainsworth et al., 2003a), represented the most appropriate single reference gene here. In comparison, a recent study on equine AMs reported *60S ribosomal protein L32* (*RPL32*) to be the most stable single reference gene (Waldschmidt et al., 2013); however that study did not include *18S* and the current study did not include *RPL32* in the list of genes assessed. Although Beekman *et al* (2011) selected a different gene combination, including *GADPH, HPRT, SDHA* and *RPL32,* in a study on equine IAD, the analyses were performed on pooled BALF-derived cells harvested from diseased horses and not isolated AMs from healthy horses, thus highlighting the potential influence of cell type and disease status on optimal housekeeping gene selection (Beekman et al., 2011).

3.2.3 LPS-induced *TNFα* and *IDO* mRNA expression in AMs and PMs

RNA was extracted from AMs and PMs at 0h and 6h following LPS (100ng/ml) stimulation, providing an average yield of 1.3µg of RNA /10^6cells (± 0.3) for AMs and 0.4µg/10^6cells (±0.01) for PMs. As TNFα secretion can be regulated at multiple levels, including the activation of TNFα-converting enzyme (Mohan et al., 2002), the possibility remained that PMs respond transcriptionally to TLR agonists, but do not release TNFα. However, consistent with the TNFα ELISA results, only AMs showed significant upregulation of *TNFα* transcript in response to LPS **(Figure 3.2.4.A)**. As shown in **Section 3.1.7**, equine AMs and PMs, like human and porcine macrophages, did not metabolise arginine to produce nitric oxide as do mouse macrophages. Instead human and porcine macrophages metabolise tryptophan through the induction of indoleamine dioxygenase (encoded by the *IDO* gene) **(Section 1.6.3)** (Silva et al., 2002, Mellor and Munn, 2004). This pathway was therefore investigated in equine macrophages. *IDO* expression was significantly upregulated in LPS stimulated AMs, but not PMs **(Figure 3.2.4.B).**

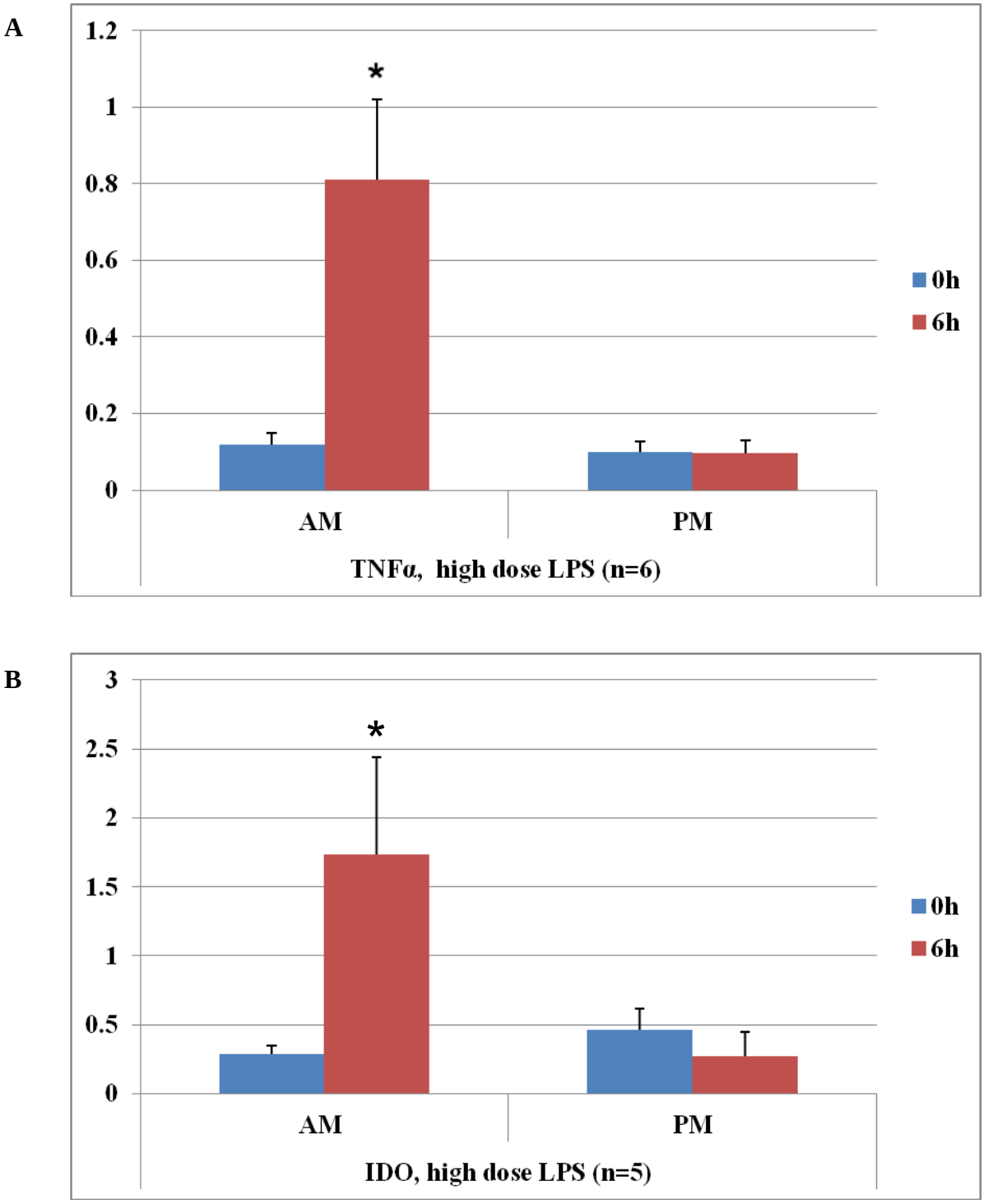


Figure 3.2.4: Relative gene expression of TNFα and IDO by LPS treated equine AMs and PMs. *Quantitative PCR of TNFα* ***(A)*** *and IDO* ***(B)*** *transcripts, respectively, were performed on cDNA samples of both AMs and PMs and mRNA expression was normalized to 18S rRNA. Results are the mean of a minimum of five experiments +/- SEM (*p<0.05 versus control 0h).*

3.2.4 CD14, CD163 and TLR4 expression in AMs and PMs

In an effort to explain the lack of LPS-responsiveness of PMs, the relative expression of various relevant cell surface markers was evaluated. These included the LPS receptor TLR4 and its co-receptor CD14. Equine CD14 antibody was recently produced by Kabithe *et al* (2010) and tested on equine PBMCs. Even though many horse studies have implied CD14 presence on AMs and PMs, its actual expression on these cells has not yet been shown (Laan et al., 2006, Morris and Moore, 1987). TLR4 is expressed by equine AMs (Waldschmidt et al., 2013). The relative expression was compared to CD163, a scavenger receptor commonly used as a macrophage marker. The anti-human AM-3K antibody against CD163 cross reacts with horse AMs, as demonstrated by immunocytochemistry (Yamate et al., 2000).

Cells were gated, as shown in **Figure 3.2.5.A** and **B.** Similar to the earlier light microscopy observations **(Section 3.1.4)**, the forward light scatter profile of the AMs suggests that they were somewhat larger on average than the PMs. AMs showed high levels of surface CD14, TLR4 and CD163 expression **(Figure 3.2.5.C-E)**. The PMs were positive for both CD163 and TLR4, whereas the expression of CD14 was negligible **(Figure 3.2.5.F-H)**. Despite the low CD14 expression by PMs, inclusion criteria for the macrophage characterisation included their ability to adhere to the plastic plates, their morphology as assessed by light microscopy and their large size and granularity (forward and side scatter, respectively) as determined by flow cytometric analysis (Grunig et al., 1991, Hawkins et al., 1998, Moore et al., 2003, Werners et al., 2004). Furthermore, upon consideration of the differential cell count of the peritoneal lavage fluid, the macrophage comprised the predominant cell population, followed by neutrophils, which could be easily distinguished by the aforementioned methods. The median fluorescence intensity from four experiments is shown in **Figure 3.2.6**.

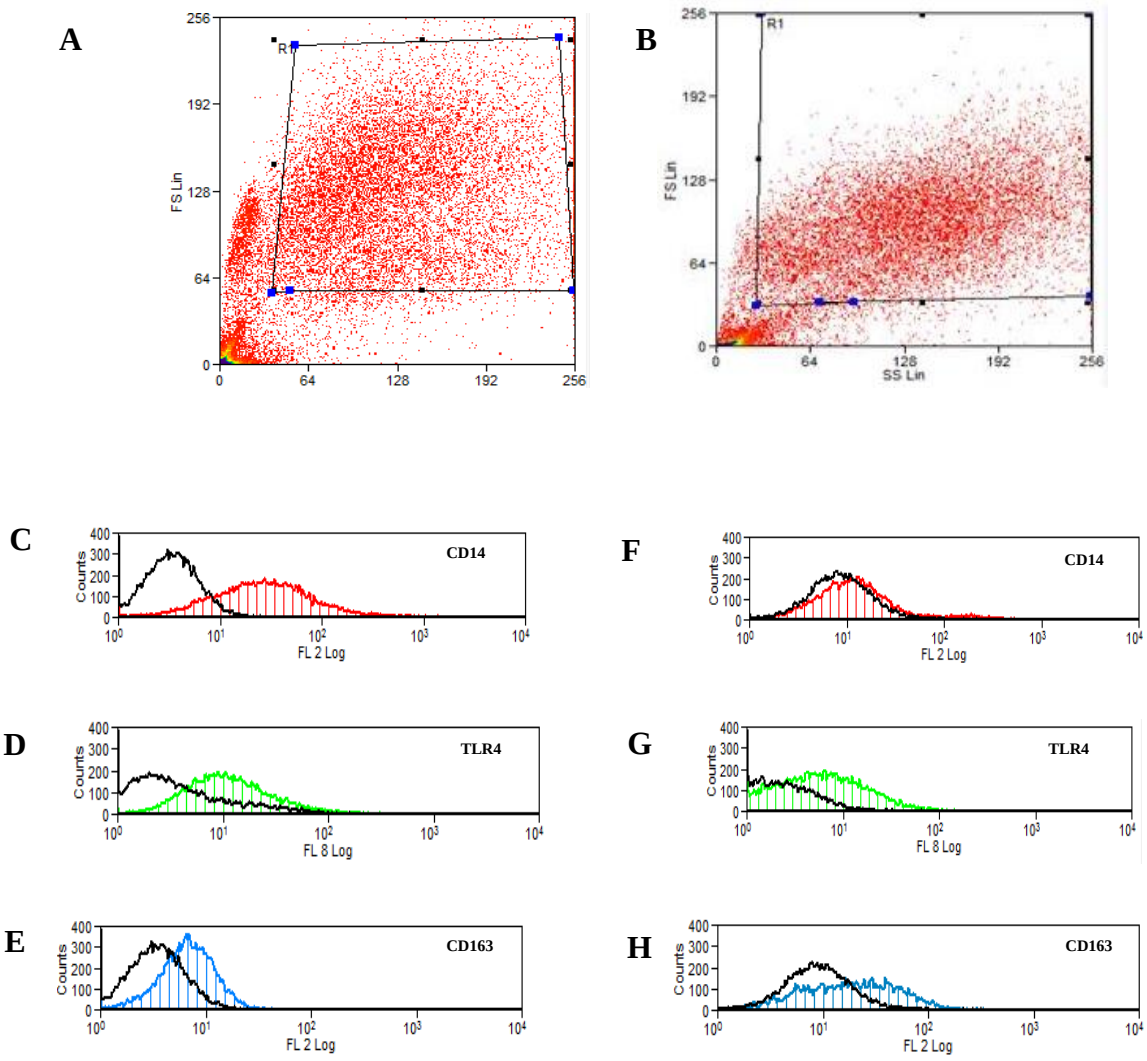


Figure 3.2.5: CD14 (C and F), CD163 (E and H) and TLR4 (D and G) expression on equine AMs and PMs.

AMs and PMs were seeded at 10^6 cells/ml and left to rest overnight. The next day nonadherent cells were washed away and cells were stained for flow cytometry. Size (FS) and granularity (SS) were used for the cell differentiation and their gating ***(A, B)****. Alveolar* ***(A)*** *and peritoneal macrophages* ***(B)*** *were stained for CD14, TLR4 and CD163. Results of AMs are shown in figures* ***C-E*** *and from PMs in figures* ***F-H****. Isotype controls are represented by a non-hatched black curve and targeting antibody is represented by a coloured hatched curve. Figures are representative of one out of a minimum of three experiments.*

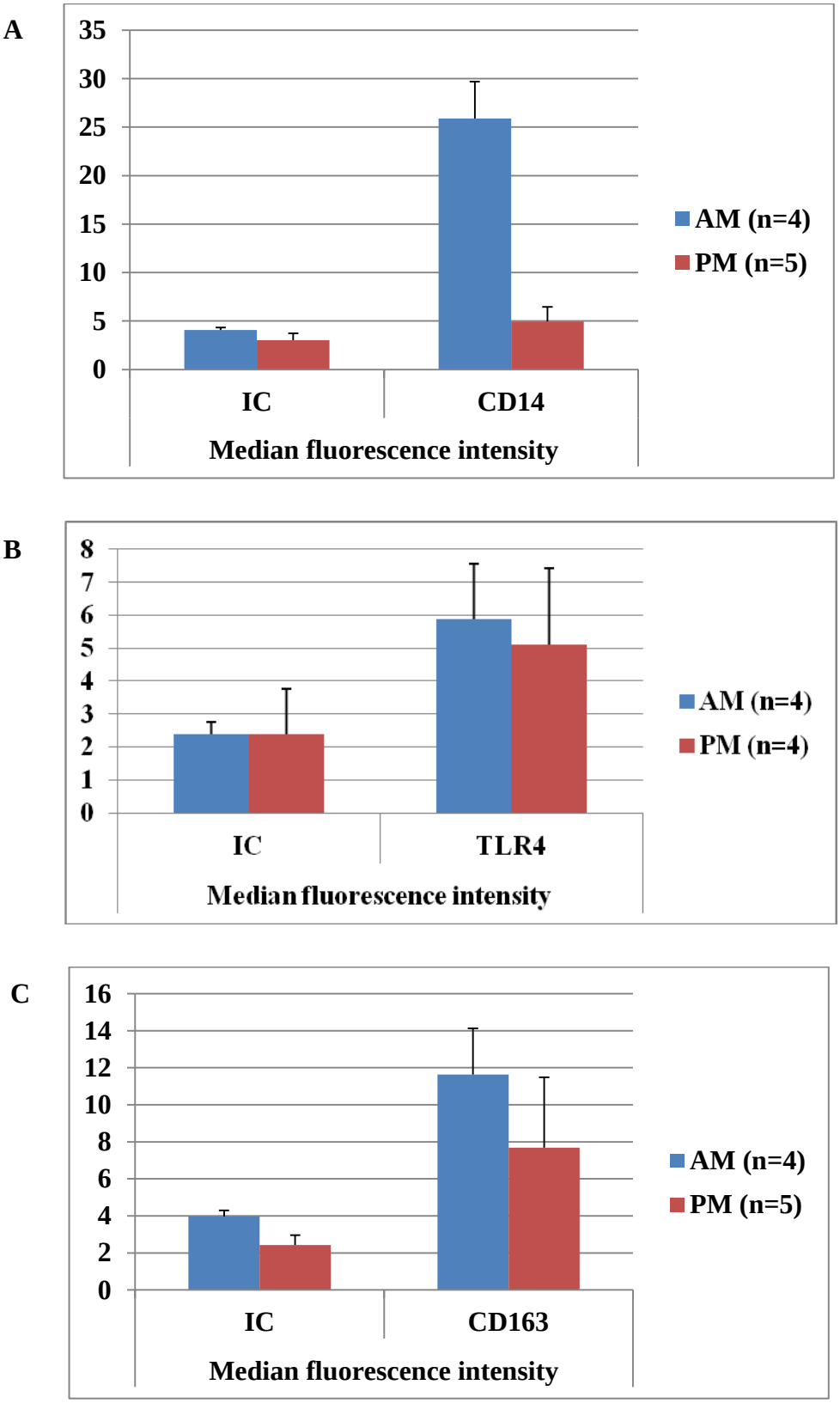


Figure 3.2.6: Median fluorescence intensity of CD14 (A), TLR4 (B) and CD163 (C) expression on equine AMs and PMs.

3.2.5 AMs show higher phagocytic activity compared to PMs

Both equine neutrophils and pulmonary alveolar macrophages have been shown to be efficient at bacterial phagocytosis (Raidal et al., 1998). The small, and less ruffled, appearance of PMs **(Section 3.1.4)** suggested that they might be less active phagocytes. **Figure 3.2.7** presents a summary of the protocol used **(Section 2.6.2)** to investigate this possibility. Compared to AMs, PMs actively bound and/or internalized a significantly lower number of FITC-labelled *E.coli*, thus indicating a relative deficiency in their phagocytic ability as shown in **Figure 3.2.8.A-B**. The average of median fluorescence of the populations (AMs and PMs) at 4°C and 37°C is presented in **Figure 3.2.8.C**.

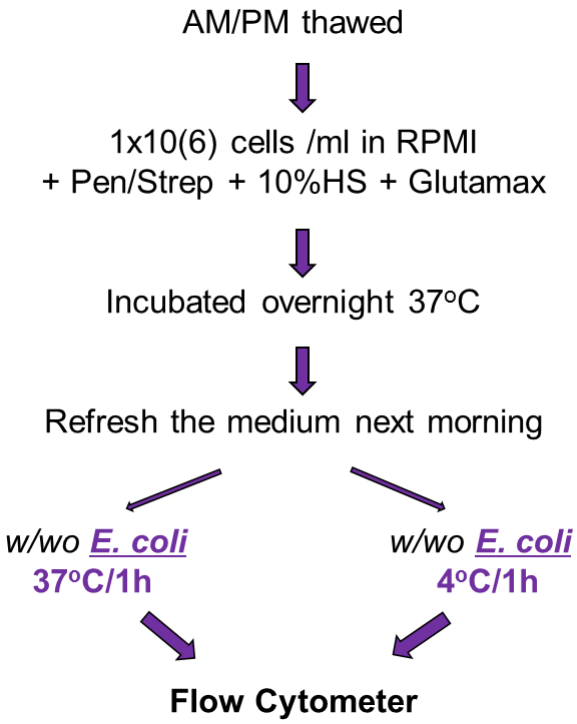


Figure 3.2.7: Experimental design of phagocytosis assay by flow cytometry on equine AMs and PMs.

Following adherence, one plate set was left at 4^0C in order to inhibit phagocytosis while the other was left at 37^0C for 30min. FITC-labeled E. coli were added into the wells for 1h. Cells were then washed twice and analysed by flow cytometry. Control cells at 37^0C and 4^0C, were included in the experiment as well as cells with E.coli at 37^0C and 4^0C.

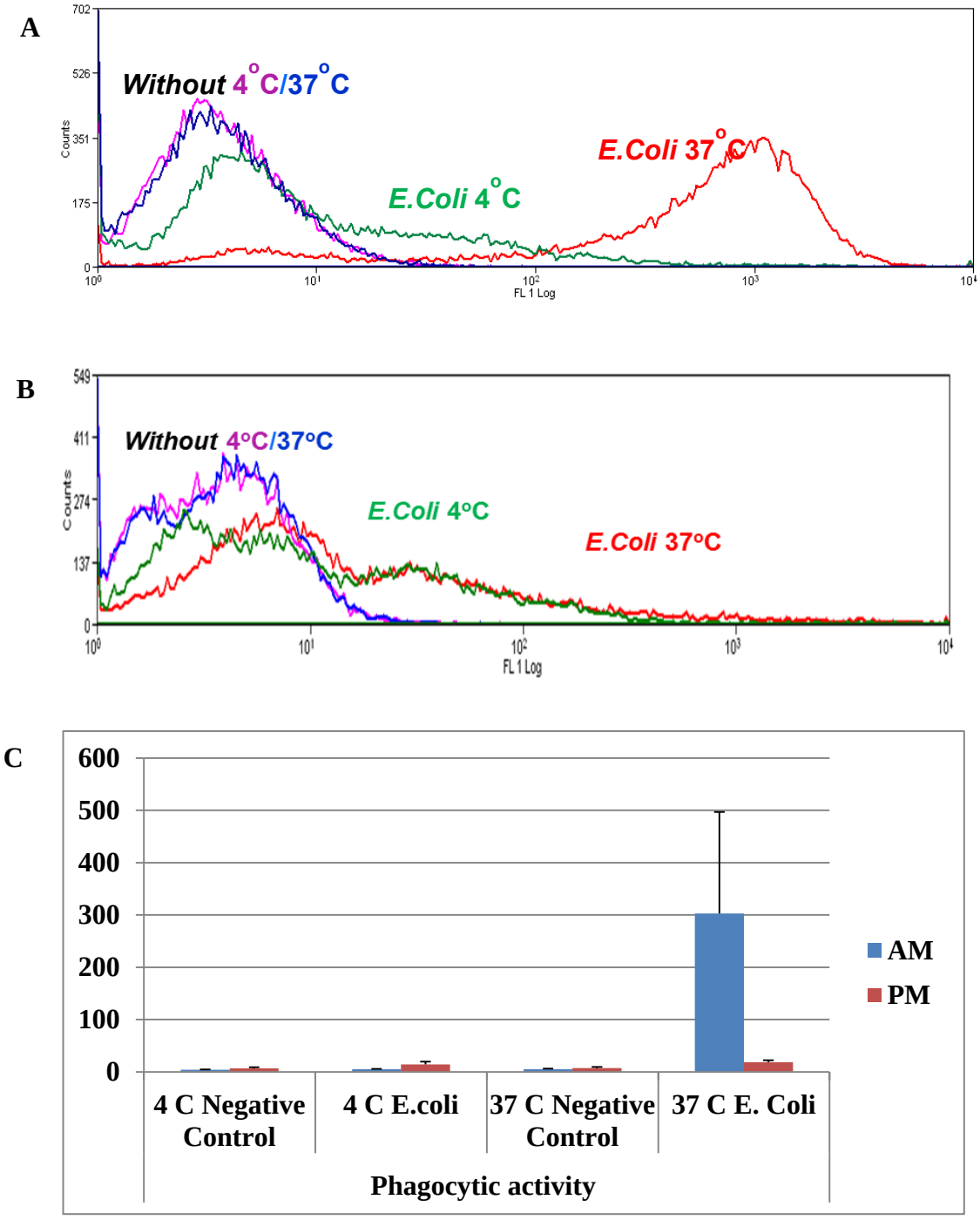


Figure 3.2.8: AMs (A), but not PMs (B), can phagocytose bacteria.

The pink and blue curves represent the fluorescence of the cells that were not treated with bioparticles of E.coli and were incubated at 4^0C and 37 ^{0}C, respectively. The green curve represents the fluorescence of the cells that were treated with bioparticles of E.coli but kept at 4^0C, while the red curve represents the fluorescence by E.coli treated cells at 37 ^{0}C. Mean of the median fluorescence intensity of three experiments +/- SEM is shown in the ***Figure 3.2.8.C****.*

3.2.6 Microarray analysis of equine AMs and PMs

As shown above, AMs and PMs differ greatly in their function and phenotype. This difference was therefore investigated on a genome-wide scale by gene expression profiling. This kind of analysis has been conducted on macrophages from other species, including humans, mice and pigs (Freeman et al., 2012, Su et al., 2002). For example, Freeman *et al* (2012), by extensive microarray analysis of numerous porcine cell and tissue samples, identified a plethora of tissue/cell type differences in gene expression and considerably improved the functional genomic annotation of the pig (Freeman et al., 2012). Microarrays have been used in equine studies but the available arrays were usually not horse-specific (Ing et al., 2004, Michelle R. Mouse et al., 2002). Graham and coworkers commenced the investigation of the horse transcriptome using human microarrays on three different equine tissues (brain, liver and articular chondrocytes) (Graham et al., 2010). Two further studies using microarray (Huang et al., 2008b) and RNA-seq (Coleman et al., 2010) investigated the equine transcriptome of different tissues and detected tissue specific expression profiles correlated with specialised tissue functions; however, these studies did not focus on differences at the level of macrophages from different origin.

In order to identify differences in gene expression between the two different types of horse macrophages, BALF (AMs) and PLF-derived (PMs) cells were plated at 10^6 cells/ml and left overnight. After nonadherent cell removal, total RNA was extracted from untreated AMs and PMs harvested from five and three horses, respectively and used for microarray analysis. As outlined in **Section 2.10**, two different types of arrays were used within the experiment, so subsequent analysis included the investigation of a possible batch effect. Both array types contained identical probes. No single sample was analysed using both array types, thus permitting the direct assessment of array type on the data generated. However, the AMs were analysed using both array types (3 with one array type and 2 with the other array type), whereas only one array type was used when analysing the PMs. Therefore, any difference between the cell types (AMs versus PMs) could not soley be attributed to a difference in the array type used. Using Partek Genomic Suite 6.6 software, any batch effect was removed and sources of variation were plotted in order to

investigate the contribution of each factor to the variation in the response variables (gene expression intensity values) across the whole arrays. As shown in **Figure 3.2.9**, following batch removal (array type) the factor with the greatest effect on gene expression intensity was the cell type (AMs / PMs). The PCA showed distinct separation of the two different groups, AMs and PMs **(Figure 3.2.10)**. No outliers were observed.

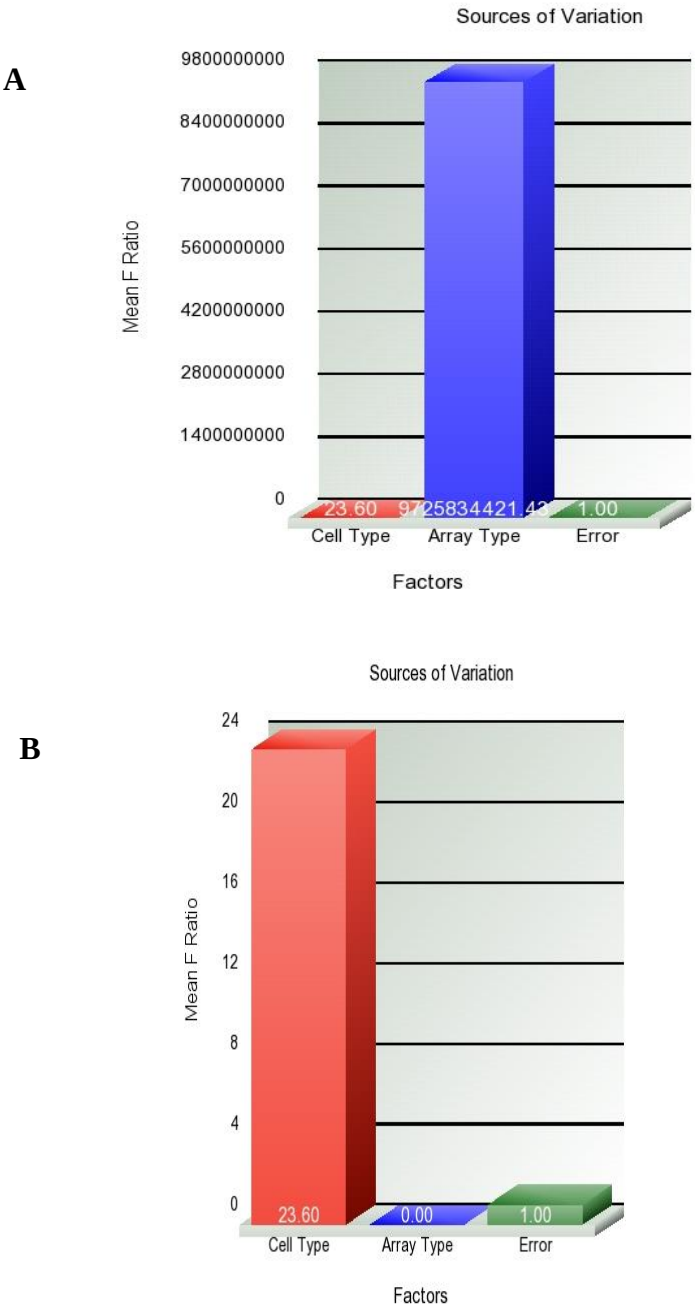


Figure 3.2.9: Sources of variation plots.

View of plots by Partek Genomic Suite 6.6 software before ***(A)*** *and after* ***(B)*** *the batch effect of array type was removed. These plots present the average effect size across all the genes for each of the factors. The x-axis shows all the factors included in the analysis. The error column represents the noise or the variability of the data not explained by the rest of the factors. The y-axis represents the mean F Ratio (signal-to-noise) of all the genes.*

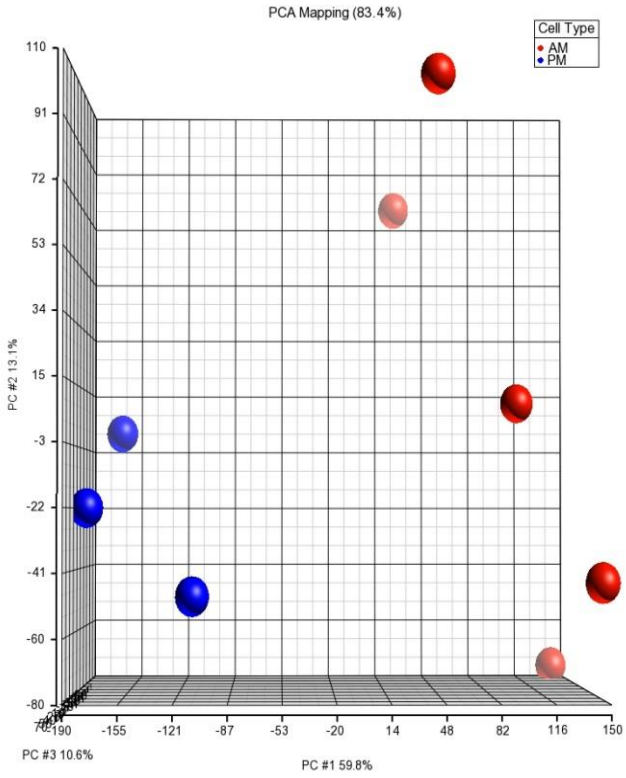


Figure 3.2.10: Principal component analysis of AMs and PMs.

Blue spheres represent the PMs and red spheres represent the AMs. The relative difference in colour intensity for each group reflects the fact that a 3-dimensional image has been transcribed onto a 2-dimensional figure. Analysis was performed by Partek Genomic Suite 6.6 software. This is a three dimensional presentation of the microarray experssion data. The proportions in each axis (PC#1, PC#2 and PC#3) represent the percentage of variance explained by each component. The percentage on the top of the graph (83.4%) represents the sum of these percentages.

A two way ANOVA test was performed in order to identify genes that were differentially expressed between the two groups. **Figure 3.2.9** depicts the factors used in the ANOVA. Of those factors, cell type was used as a fixed factor and the remainder as random factors. A list of 451 genes showing statistically-significant variance was created with a p value < 0.01 and a fold change of 9 or above, as shown in the hierarchical clustering **(Figure 3.2.11)**. However, only 25 (5.5%) of genes of

the total genelist of differentially-expressed genes were annotated, including 12/14 of those that had greater expression in AMs **(Appendix II, S1)**.

An attempt to annotate further the transcript description of the list was made, in order to gain more information regarding the genes involved in this analysis. Via the NetAffx Affymetrix database the annotation was improved to 31 genes (6.9%). From the 87 uncharacterised proteins included in the list, 31 (all upregulated in PMs) were manually annotated by reference to the human genome using the Ensembl database and via the NetAffx Affymetrix database derived sequences, as described **in Section 2.10.3 (Appendix II, S2)**. 195 genes were identified as pseudogenes and the rest were intronic normalisation controls, non-coding (nc) RNAs and small nuclear (sn) RNA or completely uncharacterised **(Table 3.2.1)**. Intronic normalization controls are used for the calculation of certain quality control metrics in Expression Console. They are labelled as "controls"; however, their sequences are derived from genes originally identified from a dataset of hybridizations representing a large number of different tissues and cell lines (www.affymetrix.com). According to Affymetrix, those probe sets showed very low signal and were therefore included in the microarray. However, it is not feasible to test their specific level of expression for every tissue type and cell population. Consequently, some of these probe sets were retained in the current experiments. The relative difference in their expression between AMs and PMs likely reflects inherent differences in these cell populations; however, such differences did not affect the interpretation of other genes that were differentially expressed when the comparisons were made.

Transcript description	No of genes	Percentage (%)
Annotated	**31**	**6.9**
Pseudogenes	195	43.3
Missing information	113	25.1
Uncharacterised proteins	87	19.3
Intronic normalisation control	13	2.7
ncRNA/snRNA	12	2.7
Total genelist	***451***	

Table 3.2.1: Summary of the annotation details of the genelist

Figure 3.2.11 shows the hierarchical clustering of the genes included in the genelist. This is a method of cluster analysis, which builds a hierarchy of clusters. In the following figure the horizontal dedongram represents the gene list, with the position of the genes relative to other genes reflecting their degree of correlation.The vertical dedongram represents the samples tested with the position of the sample relative to other samples reflecting their degree of correlation, in this case reflecting their response to LPS. Fourteen genes of the genelist had greater expression in AMs and 437 in PMs. Genes that were higher in AMs are presented in **Table 3.2.2**. Only 13 out of 437 genes that were higher in PMs were annotated and are shown in **Table 3.2.3.**, which also highlights the potential biological significance of these 13 differentially expressed genes. However, in light of the fact that the significant majority of differentially expressed genes were not annotated, it is highly likely that this list also contains genes which had a similar, if not greater, although undetermined biological significance. Due to the poor annotation of the gene list, further network analysis was performed using a different software (Biolayout *Express*^{3D}) that confirmed the results obtained here.

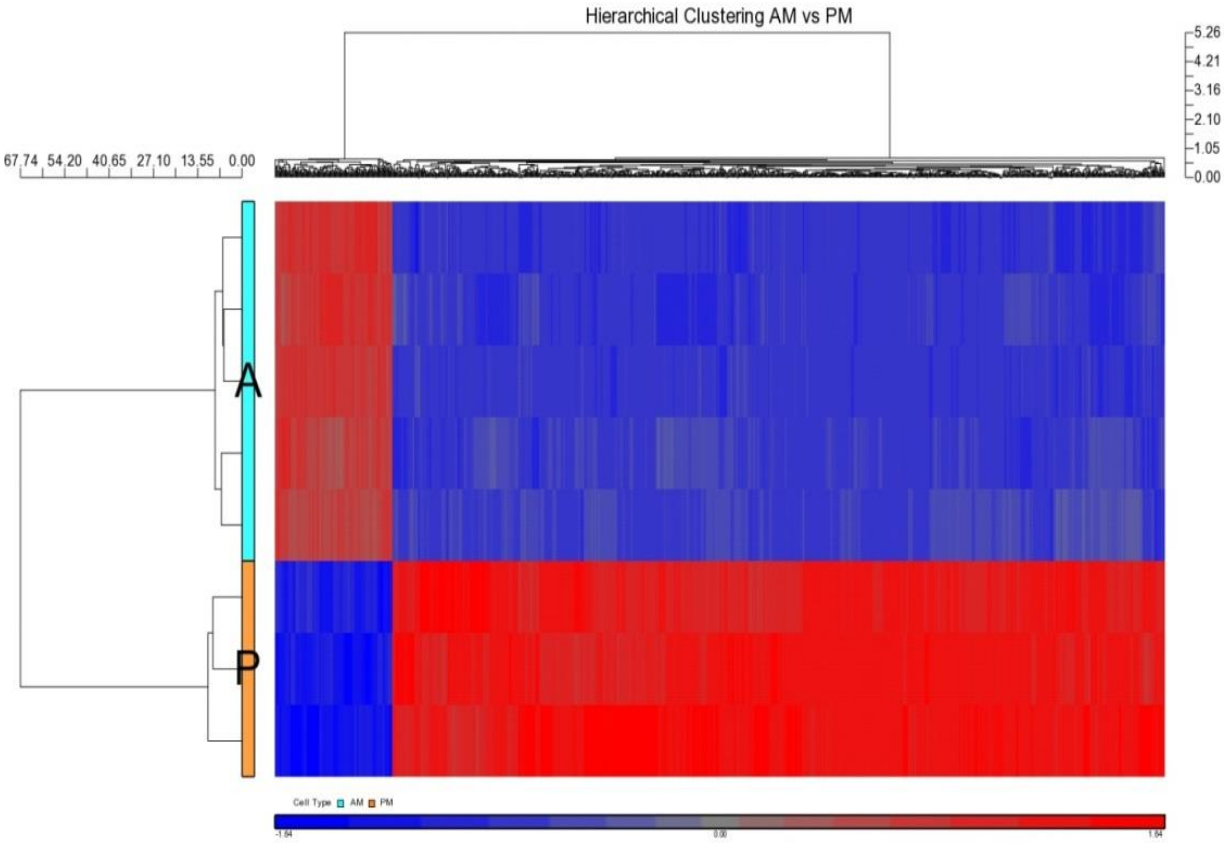


Figure 3.2.11: Hierarchical clustering of differentially expressed genes.

Hierarchical clustering of the 451 differentially expressed genes between AMs and PMs. In the main panel, the rows represent the samples, AMs are in light blue (A) and PMs in orange (P). The genes are represented in columns. In the horizontal scale, genes/probe sets with high expression are shown in red and those with low expression in blue. Those that remain stable are shown in grey. The dendrograms of the samples and genes/probe sets are shown in the left and on top of the main panel, respectively.

Transcript ID	*Gene name*	*Gene symbol*	*Biological process*	*P value*	*Differential expression*
15121321	*Cholesterol 7-α-monooxygenase-like*	*CYP7A1*	*Cholesterol homeostasis*	*1.03E-05*	*49.5*
15080122	*Platelet glycoprotein 4-like*	*CD36*	*Cell adhesion molecule-receptor for thrombospondin in platelets*	*4.54E-06*	*38.3*
15066795	*N-acylethanolamine-hydrolyzing acid amidase-like*	*NAAA*	*Degradation of bioactive fatty acid amides*	*0.0001262*	*25.7*
15088442	*Mucolipin 2*	*MCOLN2*	*Intracellular transport of membrane protein*	*0.0001424*	*21.8*
15126979	*Toll like receptor 8*	*TLR8*	*Innate and adaptive immunity*	*0.0003232*	*20.9*
14966871	*Unknown*	*Unknown*	*Unknown*	*5.99E-05*	*17.1*
15134514	*Unknown*	*Unknown*	*Unknown*	*0.0005121*	*16.2*
15051976	*Prostaglandin reductase 1*	*PTGR1*	*Metabolic inactivation of leukotriene B4*	*4.70E-05*	*14.3*
15023455	*Complement C1q subcomponent subunit B-like*	*C1QB*	*Complement component*	*1.23E-05*	*14.0*
15100625	*Phospholipase B domain containing 1*	*PLBD1*	*Defense against invading microorganisms*	*0.0002308*	*11.7*
14943095	*Early growth response 2*	*EGR2*	*Transcription factor*	*0.0004786*	*10.7*
15023462	*Complement C1q subcomponent subunit A-like*	*C1QA*	*Complement component*	*0.0003013*	*10.5*
14969419	*Catalase-like*	*CAT*	*T cell and B cell growth*	*0.0005182*	*9.5*
15023458	*Complement C1q subcomponent subunit C-like*	*C1QC*	*Complement component*	*3.13E-05*	*9.3*

Table 3.2.2: List of genes which have greater expression in AMs

The two unknown transcripts with ID 14966871and 15134514 did not blast to any known human or horse gene.

Transcript ID	*Gene name*	*Gene symbol*	*Biological process*	*p Value*	*Differential expression*
14939913	*Ig kappa chain V-III region PC 2485*	*LOC100291786*	*Unknown*	*2.15E-05*	*-29.3*
15011434	*Pentraxin 3*	*PTX3*	*Innate resistance to pathogens*	*0.000458788*	*-25.4*
14975354	*Plasminogen activator inhibitor-1*	*PAI-1*	*Ttissue homeostasis*	*0.000101477*	*-23.8*
14972820	*Olfactory receptor 476-like*	*OR476*	*Odorant receptor (potential)*	*5.30E-05*	*-17.8*
14981953	*ADAM metallopeptidase domain 19*	*ADAM19*	*Proteolytic processing of beta-type neuregulin isoforms*	*0.000570202*	*-16.3*
15060218	*Lumican*	*LUM*	*Major keratan sulfate proteoglycan*	*0.000107293*	*-13.9*
15053644	*Calponin-3-like*	*CNN3*	*Modulation of smooth muscle contraction*	*0.000464655*	*-12.9*
15076479	*Sema domain, immunoglobulin domain (Ig), short basic domain*	*SEMA3C*	*Regulation of developmental processes*	*0.00019507*	*-11.5*
15030285	*Olfactory receptor 2W1-like*	*OR2W1*	*Odorant receptor (potential)*	*0.00020208*	*-11.4*
15030212	*Histone H2A type 1-like*	*HIST1H2AG*	*Core component of nucleosome*	*0.000478705*	*-11.0*
15094905	*Chemokine (C-X-C motif) receptor 7*	*CXCR7*	*Activation of MAPK signaling pathway*	*0.000278447*	*-10.3*
15120364	*Transcription factor 7-like 2 (T-cell specific, HMG-box)*	*TCF7L2*	*Wnt signaling pathway and modulates MYC expression*	*2.63E-05*	*-9.7*
15103641	*microRNA mir-7-2*	*MIR7-2*	*Post-transcriptional regulation of gene expression*	*2.97E-05*	*-9.7*

Table 3.2.3: List of annotated genes which have greater expression in PMs

Network analysis of the same normalised data was performed using Biolayout *Express*^{3D} software, with a MCL inflation value of 2.2, a Pearson correlation coefficient threshold (r) of 0.93 and the smallest cluster was set at three nodes. Transcripts with dynamic range less than 1.5 were removed from the analysis. The graph created 1,321 clusters consisted of a total number of 12,341 nodes, connected with 1,947,031 edges and provided a more in depth understanding of the gene expression profiles of the two cell types. Consistent with the results of the PCA, the network analysis **(Figure 3.2.12)** clearly distinguished two groups of clusters; one consisting of gene clusters with greater expression in AMs and one consisting of gene clusters with greater expression in PMs. The remainder of the genes showed similar expression in both cell types. Sample to sample variation within each group was not apparent when analysing the data using Biolayout express software to identify differentially expressed gene clusters. Due to the high number of clusters created (1,321) it was not feasible to present or critically interpret all of the data generated. Therefore more detailed analysis was targeted towards the 5 clusters of differentially expressed genes which contained the highest number of genes. The top clusters of transcripts with the greatest expression in AMs compared with PMs and PMs compared with AMs were numbers 2, 3, 4 and 5; and 1, 8, 15, 17 and 25, respectively. The numerical figure used to identify each cluster is inversely proportional to the number of genes within the cluster. For example, cluster 1 contains the greater number of genes. Total gene lists of these clusters are presented in **Appendix II**, **supplementary data S3** (total gene number: 3,212) and **S4** (total gene number: 773) respectively. Genes previously identified using the Partek software as showing statistically significant differential expression between the 2 groups **(Tables 3.2.2 and 3.2.3)** were also identified within the gene clusters highlighted using the Biolayout Express software.

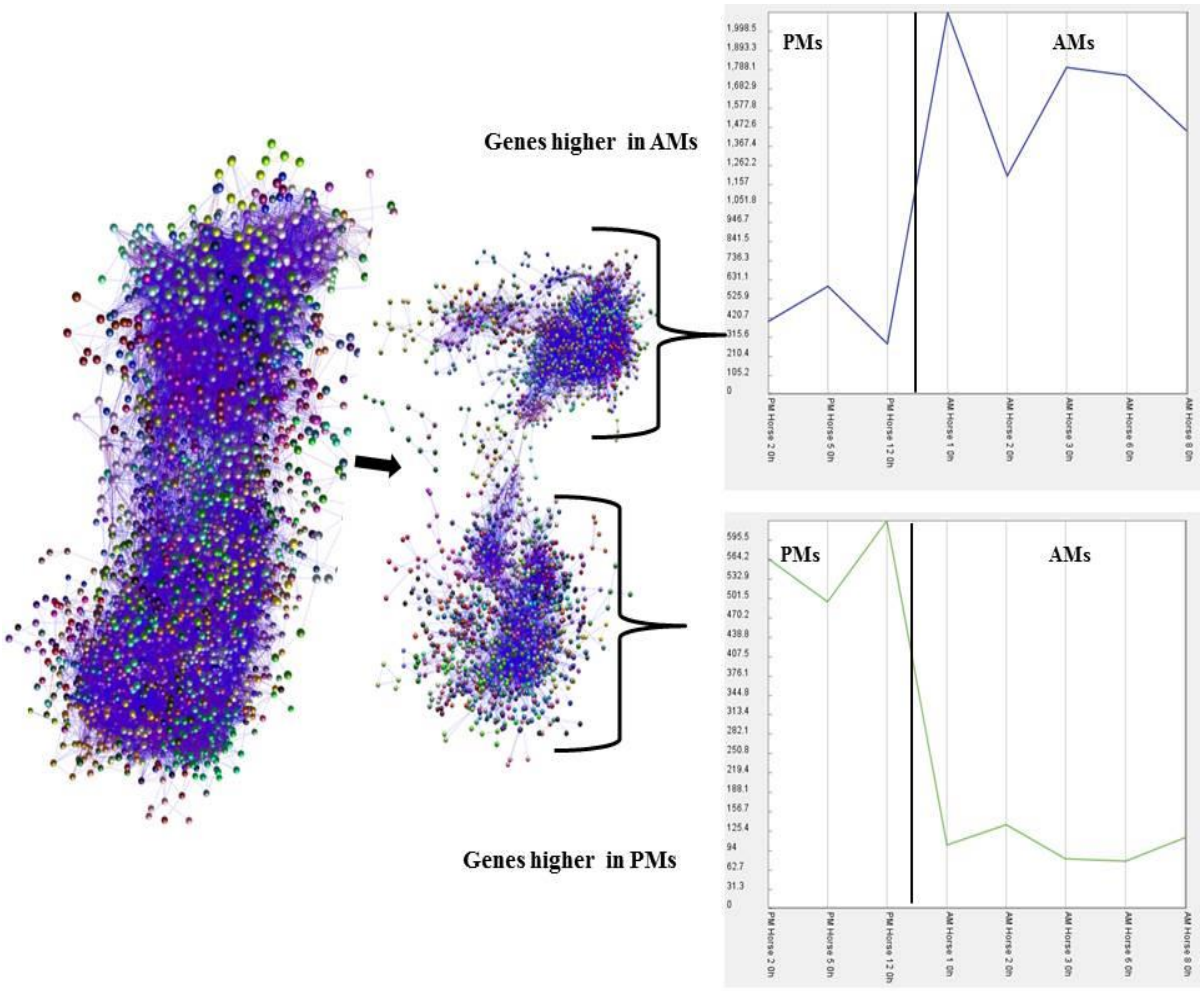


Figure 3.2.12: Network analysis of genes differentially expressed in AMs and PMs.

Graph was performed by the Biolayout Express^{3D} software (MCL 2.2, r=0.93). Nodes with the same colour belong to the same cluster of co-expressed genes. On the left panel is shown the total graph created and in the middle, the clusters with the two different expression profiles. On the right top is shown the expression profile of the genes more highly expressed in AMs and on the bottom right those more highly expressed in PMs. The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 2, 5 and 12 were included in the PM group whereas horses 1, 2, 3, 6 and 8 in the AM group, as depicted in order (left to right) on the x-axes.

Analysis of the genelists derived from clusters with greater expression in AMs demonstrated enhanced expression of genes involved in cellular metabolic processes, leukocyte/myeloid cell differentiation, immune response, cellular differentiation/development and RNA metabolic processes (Huang et al., 2008a, Huang et al., 2009). In particular, the genelists more highly expressed in AMs included genes specifically-expressed by macrophages or encoding proteins highly involved in macrophage differentiation, such as *CSF1* and its receptor *CSF1R*, colony stimulating factor 2 receptor beta (*CSF2RB*), members of the scavenger receptor family [*CD14, CD68*, *CD163*, macrophage scavenger receptor 1 (*MSR1*)], sialic acid-binding immunoglobulin-type lectins (*SIGLEC1*), *CD80*, monocyte to macrophage differentiation protein (*MMD*), myeloid cell nuclear differentiation antigen (MNDA), cell differentiation protein RCD1 homolog (*RCD1*), forkhead box protein P1 (*FOXP1*), schlafen 5 (*SLFN5*) and transforming growth factor beta 1 (TGFB1) **(Figure 3.2.13)**.

Moreover, of transcripts that showed relatively greater expression in AMs, many are clearly related to immune response. This included genes such as Mannose receptor C type 1 (*MRC1*), genes that play a role in the NFKB signalling pathway [Mitogen-activated protein kinase kinase kinase 2 (*MAP3K2*), Interleukin-1 receptor-associated kinase 4 (*IRAK4*)], several pattern recognition receptors (*TLR3*, *TLR6*, *TLR7*, *TLR8*), members of the TNF and TNFRS receptor superfamily (TNFRSF) (*CD40*, *TNFRSF1A*, *TNFRSF13B*), interleukin *IL18* and interleukin receptors (*IL6R*, *IL17R*), as well as suppressors of cytokine signaling (*SOCS4*, *SOCS6*) **(Figure 3.2.14)**.

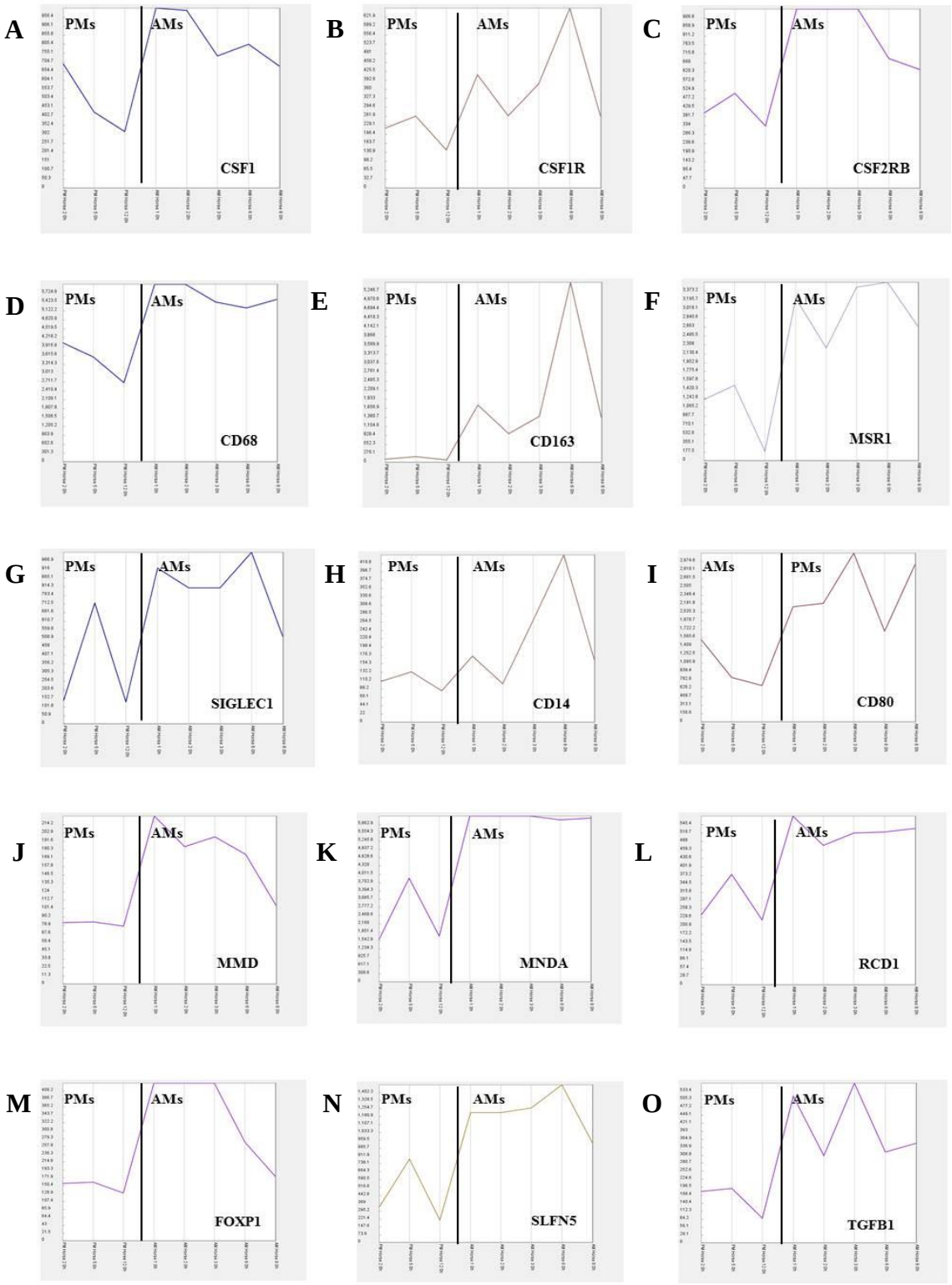


Figure 3.2.13: Expression profile of genes involved in macrophage differentiation.

Selection of genes more higly expressed by AMs. These include genes which were either (a) located within the top 5 clusters of genes with higher expression in AMs (Clusters 2, 3, 4) or (b) located

within other smaller clusters, yet with a similar expression profile and considered to have potential biological significance (e.g. CSF1, CD68, SIGLEC1, CD80, MMD, MNDA). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 2, 5 and 12 were included in the PM group whereas horses 1, 2,3, 6 and 8 in the AM group, as depicted in order (left to right) on the x-axes.

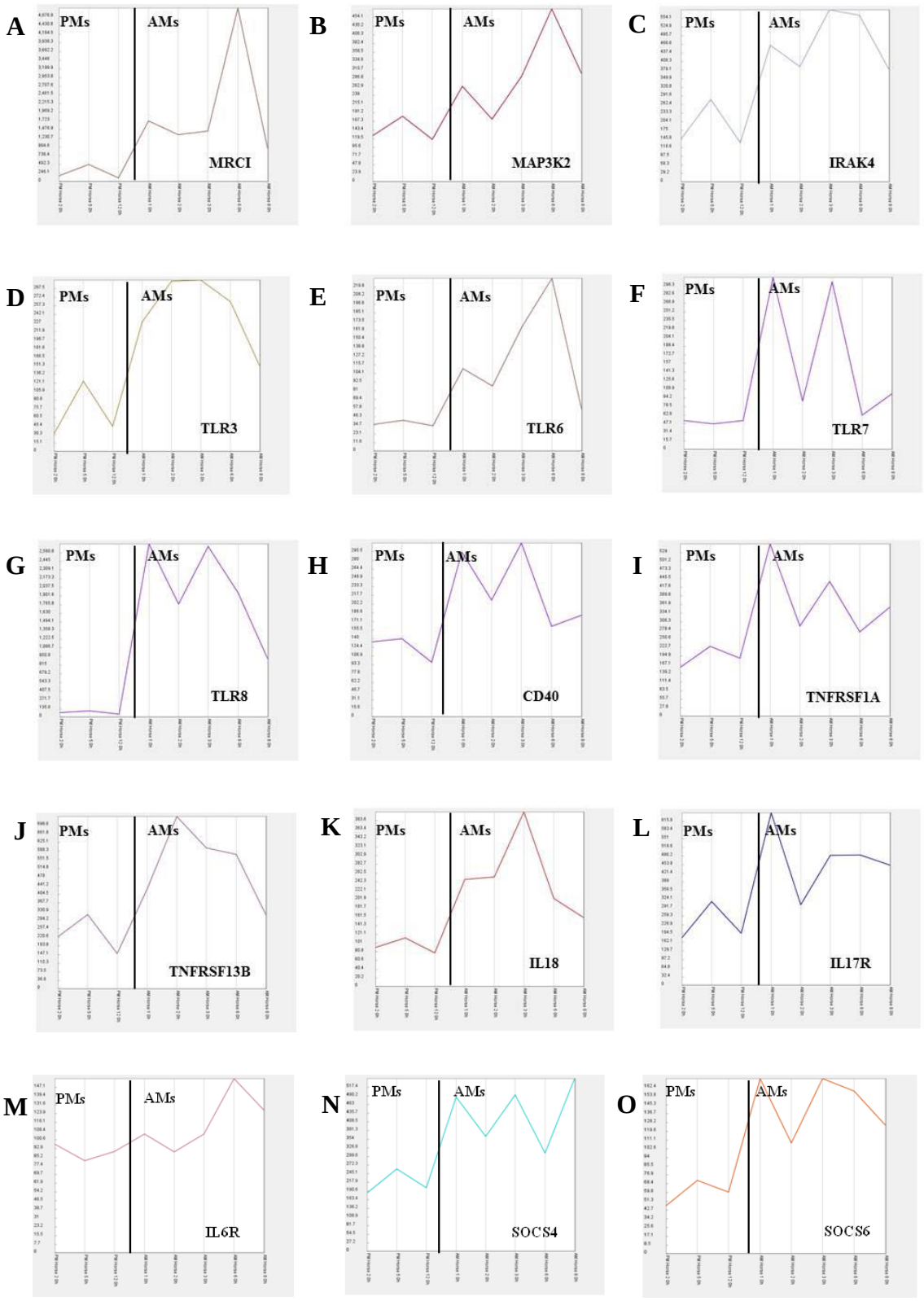


Figure 3.2.14: Expression profile of immune associated genes.

Selection of genes presented here were more higly expressed by AMs These include genes which were either (a) located within the top 5 clusters of genes with higher expression in AMs (Clusters 2, 3, 4) or (b) located within other smaller clusters, yet with a similar expression profile and considered to have potential biological significance (e.g TLR3, TNFRSF13B, IL18, SOCS4, SOCS6). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 2, 5 and 12 were included in the PM group whereas horses 1, 2,3, 6 and 8 in the AM group, as depicted in order (left to right) on the x-axes.

Analysis of the genelists derived from clusters with greater expression in PMs (Cluster 1 expression profile) demonstrated enhanced expression of genes involved in inflammatory/defense and immune response, intracellular signalling cascade, cellular metabolic processes and multicellular organismal development. Genelists of clusters higher in PMs included several chemotactic chemokines (*CXCL1*, *CXCL3*, *CXCL6*), chemokine receptor 7 (*CXCR7*), both pro and anti-inflammatory cytokines (*IL1A*, *IL1B, IL6*, *IL8, IL10*, *IL11*, *IL23A*) and interleukin receptors (*IL1R*, *IL1RN*, *IL2RA,IL4R*) **(Figure 3.2.15)**.

Additionally, the high expression of the acute phase protein pentraxin 3 (*PTX3*), *NFκB, TLR9* and of several interferons (*IFNA1*, *IFNA2*) **(Figure 3.2.16.A-E respectively)** suggests that PMs are more activated compared to AMs, which might explain why the cells did not respond to several stimuli (LPS, Poly IC and heat killed bacteria of *S. typhimurium*). In effect, the PM cells could be considered LPS-tolerant, due to prior exposure to an LPS-like stimulus. Genes encoding vascular endothelial growth factor A (*VEGFA*) and one of its receptors (*FLT1*), which play an important role in angiogenesis and vasculogenesis, were also observed in cluster 1 and showed relatively greater expression in PMs, compared to AMs **(Figure 3.2.16.F and G)**. In the same cluster, *CSF3* and its receptor were identified **(Figure 3.2.16.H and I)**.

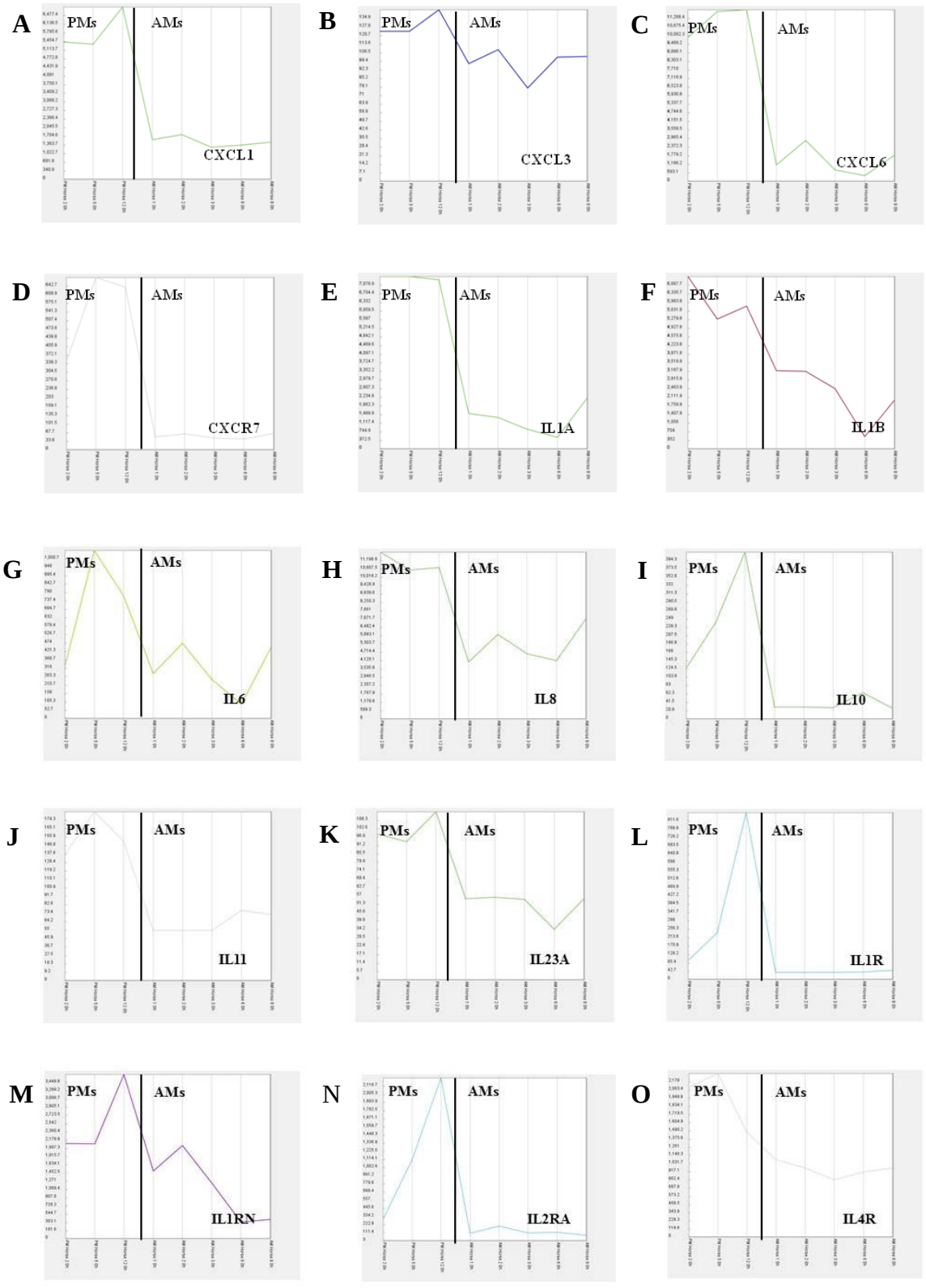


Figure 3.2.15: Expression profile of inflammatory related genes

Selection of genes presented here were more higly expressed by PMs. These include genes which were either (a) located within the top 5 clusters of genes with higher expression in PMs (e.g. Clusters 1 and 15) or (b) located within other smaller clusters, yet with a similar expression profile and considered to have potential biological significance (e.g IL6, IL1RN and IL2RA). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 2, 5 and 12 were included in the PM group, whereas horses 1, 2,3, 6 and 8 in the AM grou, as depicted in order (left to right) on the x-axes.

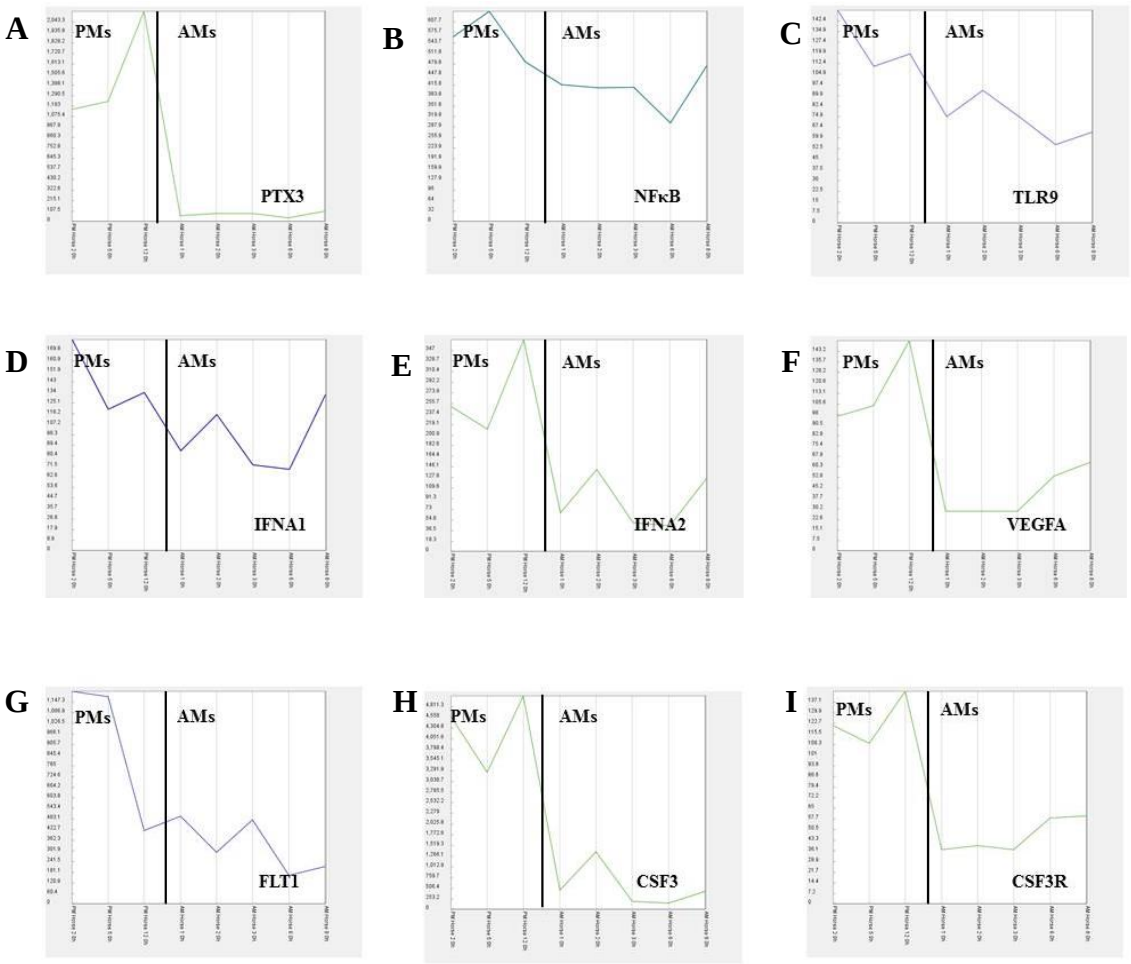


Figure 3.2.16: Expression profile of immune associated genes (A-E), genes regulating angiogenesis (F-G) and cell differentiation-proliferation (H-I).

Selection of genes presented here were more higly expressed by PMs. These include genes which were either (a) located within the top 5 clusters of genes with higher expression in PMs (e.g. Clusters 1 and 17) or (b) located within other smaller clusters, yet with a similar expression profile and considered to have potential biological significance (e.g NFKB, TLR9, IFNA2, FLT1). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 2, 5 and 12 were included in the PM group whereas horses 1, 2,3, 6 and 8 in the AM group, as depicted in order (left to right) on the x-axes.

3.3 Gene expression after LPS stimulation in equine AMs and their comparison with human LPS treated AMs

As stated in the introduction **(Section 1.5)**, AMs represent the principal cells responsible for clearance of particulate material within the lungs (Dixon PM and McGorum, 1997). Under normal conditions, they neither attack nor trigger other host cells; rather they eliminate and degrade inhaled particles and microorganisms via phagocytosis (Dixon PM and McGorum, 1997). When exposed to pathogens or antigens they become activated and induce inflammation (Dixon PM and McGorum, 1997). In the horse, common infectious organisms such as *Streptococcus zooepidemicus*, *Streptococcus pneumoniae*, *Pasteurella/Actinobacillus* species may present as opportunistic pathogens, which trigger the equine respiratory immune system and induce inflammation (Wood et al., 1993, Burrell et al., 1996). Lipopolysaccharide, derived from gram negative bacteria, is considered a major factor for the induction of inflammation in the airways via the activation of macrophages, neutrophils and endothelium of various vascular beds (Andonegui et al., 2003). Interestingly, horses and humans are more sensitive to endotoxin induced cardio-pulmonary shock and have a high endotoxaemia-associated mortality compared to other mammalian species including rodents (Berczi et al., 1966, Warren et al., 2010, Werners et al., 2005). Although the precise mechanism for this phenomenon is not completely understood, equine pulmonary intravascular macrophages are able to endocytose LPS and produce TNFα and IL1β and depletion of pulmonary intravascular macrophages decreases the pulmonary immune response to LPS (Parbhakar et al., 2005).

Most previous studies of equine macrophage gene expression were based on reverse transcription quantitative polymerase chain reaction (RT qPCR), an approach restricted by the limited number of genes which can be assessed in a single assay (Moore et al., 2003, Jackson et al., 2004). Microarrays have rapidly become a widely used tool as they enable the simultaneous assessment of the expression of a large number of genes and provide a vast amount of information on diverse biological processes. The present study investigates gene expression in equine LPS treated AMs

using a whole transcript horse specific microarray. **Figure 3.3.1** summarises the experimental protocol used for the analysis of the microarray data.

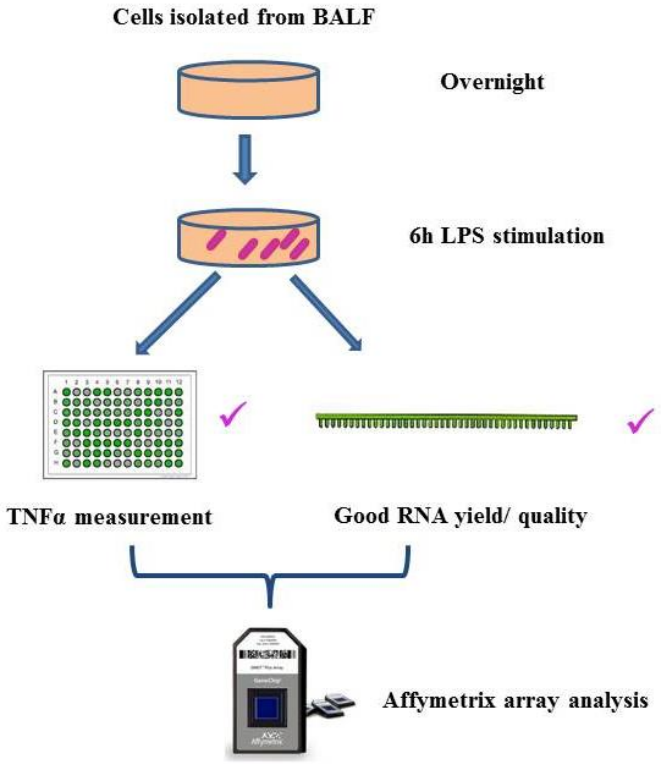


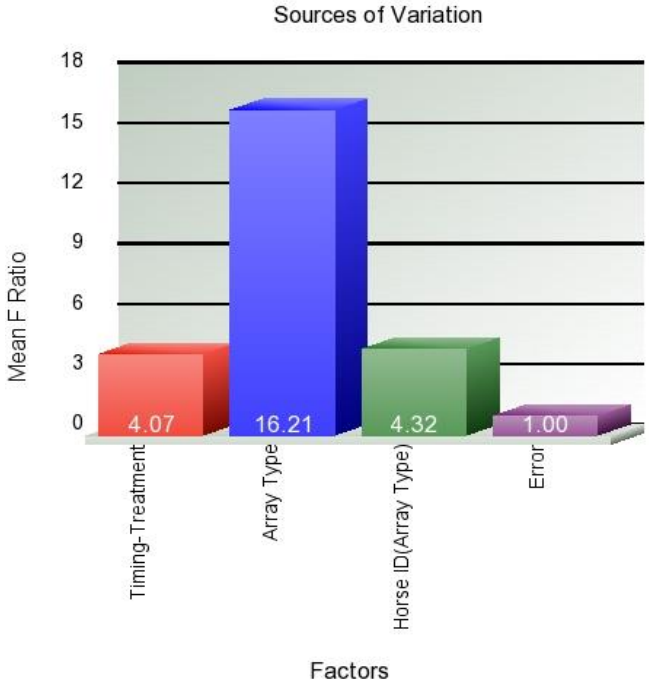
Figure 3.3.1: Workflow for gene expression study in LPS treated horse AMs.

BALF-derived cells were plated at 10^6 cells/ml and incubated overnight. The following morning, supernatant was removed and adherent cells (AMs) were stimulated with LPS for 6h. Prior to microarray analysis, RNA yield and quality were checked and confirmation of an LPS-induced response was obtained by the measurement of TNFα in cell culture supernatants. Negative controls comprised cells which were cultured in the same way but not stimulated with LPS.

As with the comparison of AMs and PMs described above, a batch effect due to the different array types was also observed in this analysis **(Figure 3.3.2.A)**. Using Partek Genomic Suite 6.6 software, the batch effect was removed and sources of variation were plotted in order to investigate the contribution of each factor to the variation in the response variables (gene expression intensity values) across the whole arrays **(Figure 3.3.2.B)**. Following removal of the batch effect (array type), both LPS treatment and the individual horse greatly influenced differences in gene expression.

No outliers were observed and PCA classified the samples into two evidently different groups; one consisted of untreated and the other of LPS treated AMs **(Figure 3.3.3)**. By using Partek software a gene list of 240 genes was created ($p<0.05$). Of those genes, 220 were upregulated in LPS treated AMs and 20 were downregulated as shown in the hierarchical clustering **(Figure 3.3.4)**. 207 out of 240 (86.2%) genes were already annotated in the Affymetrix annotation file imported in Partek software. The remainder of the genes were identified by using the Affymetrix database, NetAffx (www.affymetrix.com) **(Appendix III, S1)**.

A



B

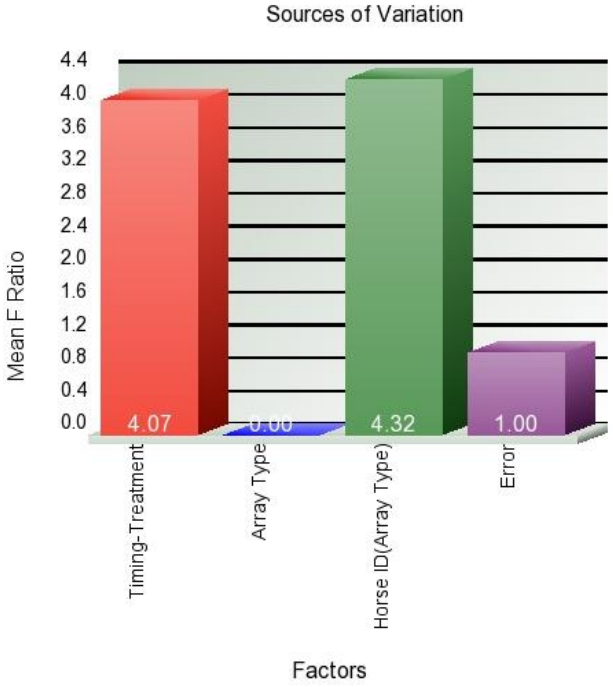


Figure 3.3.2: Sources of variation plots.

View of plots by Partek Genomic Suite 6.6 software before ***(A)*** *and after the batch effect of array type was removed* ***(B)****. These plots present the average effect size across all the genes for each of the factors (x-axis). The error column represents the noise or the variability of the data not explained by the rest of the factors. The y-axis represents the mean F Ratio (signal-to-noise) of all the genes.*

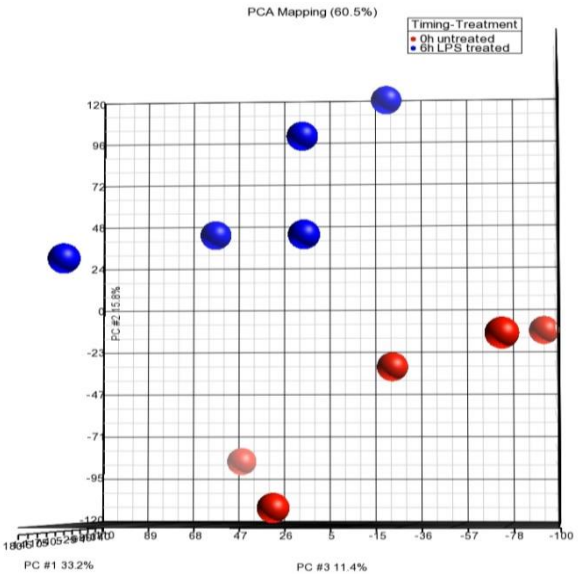


Figure 3.3.3: Principal component analysis of LPS treated and untreated AMs.

The relative difference in colour intensity for each group reflects the fact that a 3-dimensional image has been transcribed onto a 2-dimensional figure.Analysis was performed by Partek Genomic Suite 6.6 software. This is a three dimensional presentation of the microarray experssion data. The proportions in each axis (PC#1, PC#2 and PC#3) represent the percentage of variance explained by each component. The percentage on the top of the graph (60.5%) represents the sum of these percentages.

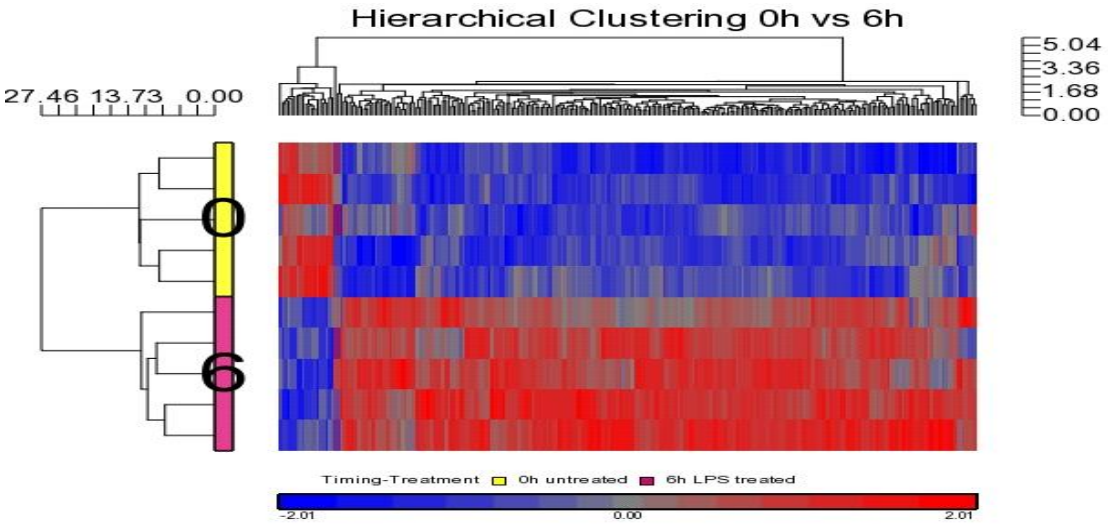


Figure 3.3.4: Hierarchical clustering of differentially expressed genes in LPS treated and untreated AMs.

Hierarchical clustering of the 240 differentially expressed genes between LPS treated AMs and controls. In the main panel, the rows represent the samples. LPS treated AMs are those in pink (6h) and the untreated AMs (controls, 0h) in yellow. The genes are represented in columns. In the horizontal scale, genes/probes with high expression are shown in red and those with low expression are shown in blue. Those that remain stable are shown in grey. The dendrograms of the samples and genes/transcripts are shown in the left and on top of the main panel.

Numerous well-known inflammation- related genes were upregulated in LPS treated AMs **(Figure 3.3.5)**. Based upon the gene ontology tool of the Partek Genomic Suite 6.6 package, the principal biological process categories were identified as "inflammation" and "cell defence" **(Figure 3.3.6)**. Genes involved in apoptosis were also detected, a phenomenon that has been described before in humans and mice (Joshi et al., 2003). The genes downregulated at 6h following LPS stimulation are shown in **Table 3.3.1**.

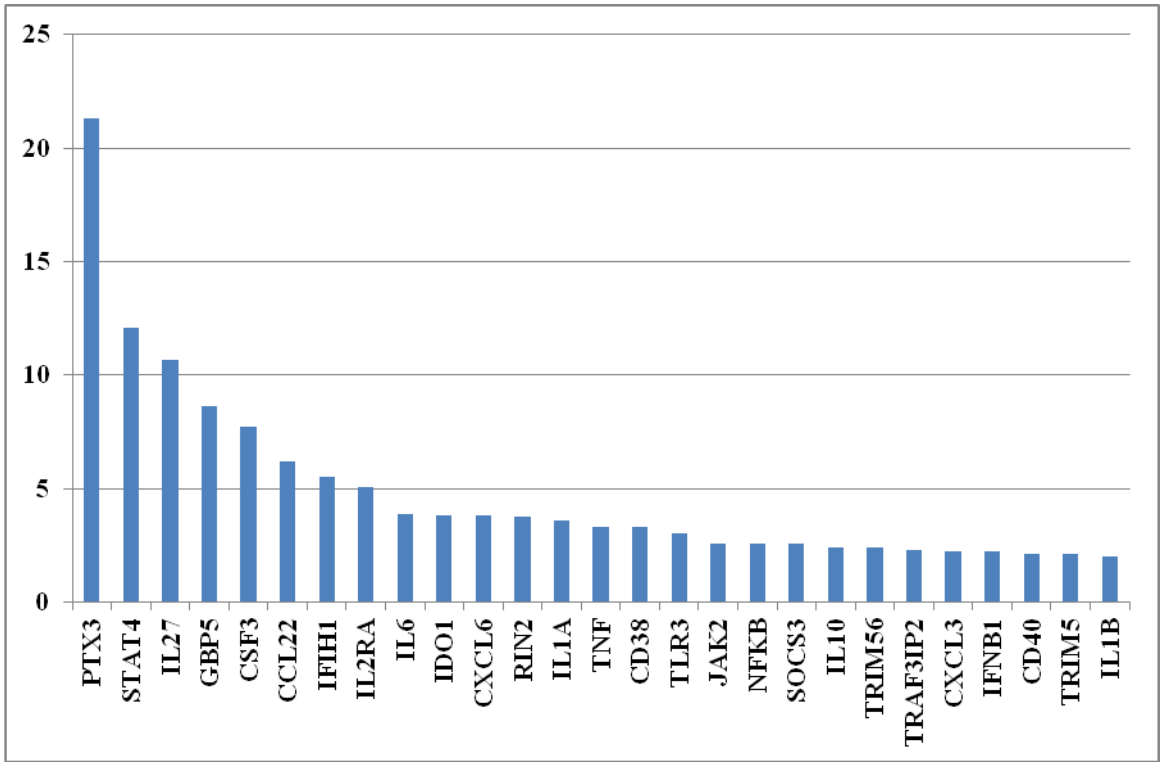


Figure 3.3.5: Fold change of inflammatory related genes upregulated after LPS treatment.

The y-axis shows the fold change of a list of inflammation related genes upregulated in LPS treated AMs at 6h. PTX3, pentraxin 3; STAT4, Signal Transducer and Activator of Transcription; IL27, Interleukin 27; GBP5, guanylate binding protein 5; CSF3, colony stimulating factor 3; CCL22, C-C motif chemokine 22; IFIH1, Interferon induced with helicase C domain 1; IL2RA, Interleukin 2 receptor alpha; IL6, Interleukin 6; IDO1, indoleamine 2,3-dioxygenase 1; CXCL6, chemokine (C-X-C motif) ligand 6; RIN2, Ras and Rab interactor 2; IL1A, Interleukin 1 alpha; TNF, tumor necrosis factor; CD38, cluster of differentiation 38; TLR3, Toll like receptor 3; JAK2, Janus kinase 2; NFKB, nuclear factor kappa-light-chain-enhancer of activated B cells; SOCS3, suppressor of cytokine signaling 3; IL10, Interleukin 10, TRIM56, tripartite motif containing 56; TRAF3IP2, TRAF3 Interacting Protein 2; CXCL3, chemokine (C-X-C motif) ligand 3; IFNB1, Interferon beta 1; CD40, cluster of differentiation 40; TRIM5, tripartite motif containing 5; IL1B, Interleukin 1 beta .

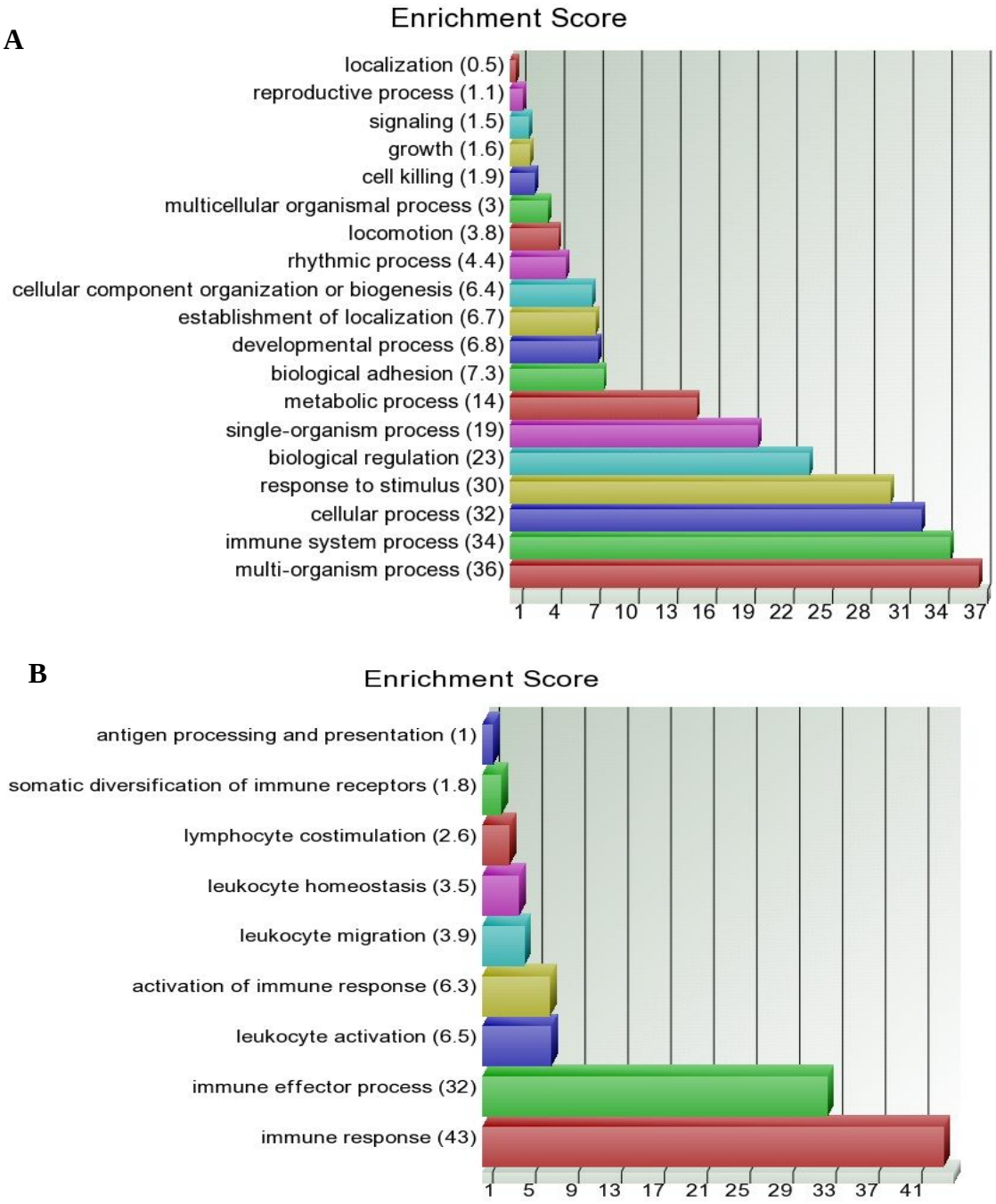


Figure 3.3.6: Main gene functional groups induced by LPS treatment in horse AMs.

Gene ontology analysis tool from the Partek Genomic Suite software, was used. The histogram shows the main gene functional groups affected by LPS stimulation ***(A)*** *and in particular the immune related biological processes that the upregulated genes are involved are shown in* ***(B)****.*

Transcript ID	*Gene name*	*Gene symbol*	*Biological process*	*P value*	*Differential expression*
14954767	**Neutral amino acid transporter B(0)-like**	***SLC1A5***	**Cell surface receptor**	**0.00800835**	**-2,00761**
14957654	**Serine/threonine-protein kinase Sgk1-like**	***SGK1***	**Apoptosis**	**0.00319022**	**-2,02089**
14945010	**Apoptosis enhancing nuclease**	***AEN***	**Regulation of apoptosis**	**0.00203566**	**-2,06747**
14971846	**Patatin-like phospholipase domain-containing protein 2-like**	***PNPLA2***	**Regulation of cellular process**	**0.00800983**	**-2,07838**
15068182	**Wolframin-like**	***WFS1***	**Regulation of apoptosis**	**0.00425089**	**-2,08333**
14967281	**Solute carrier family 46 (folate transporter), member**	***SLC46A1***	**Intestinal proton-coupled high-affinity folate and heme transporter**	**0.00407444**	**-2,12738**
15026677	**RIO kinase 1 (yeast)**	***RIOK1***	**Unknown**	**0.00510916**	**-2,14655**
14932187	**Intronic normalization control**			**0.00630691**	**-2,25422**
15064847	**Phospholipase A2, Group XV**	***PLA2G15***	**Transacylase and calcium-independent phospholipase A2 activity**	**0.00253817**	**-2,34886**
15126975	**Toll like receptor 7**	***TLR7***	**Immune defense/ response**	**0.00711464**	**-2,38297**
15095766	**Protein tyrosine phosphatase, non-receptor type 6**	***PTPN6***	**Apoptosis**	**0.00128212**	**-2,45451**
14972866	**Leupaxin**	***LPXN***	**Signal transduction**	**0.00169939**	**-2,46798**
15106911	**Arrestin, beta 1**	***ARRB1***	**Signal transduction**	**0.00635258**	**-2,47078**
15094354	**2-acylglycerol O-acyltransferase 1-like**	***MOGAT1***	**Glycerol metabolism-Lipid biosynthesis/metabolism**	**0.00101971**	**-2,59006**
15073546	**Regulator of G-protein signaling 2,**	***RGS2***	**Regulation of cellular process**	**0.00796698**	**-2,61257**
15071057	**Proepiregulin-like**	***EREG***	**Regulation of cellular process**	**0.00027312**	**-2,7328**
14998968	**Peroxisome proliferator-activated receptor gamma-like**	***PPARG***	**Immune defense/response and system process – anti-inflammatory action**	**0.00176408**	**-2,88641**
15032122	**Glutamate-cysteine ligase, catalytic subunit**	***GCLC***	**Regulation of apoptosis - regulation of cellular process**	**0.00129169**	**-2,90054**
15124723	**Src-like-adapter-like**	***SLA***	**Negatively regulates T-cell receptor signaling.**	**0.00677987**	**-3,12657**
15015651	**lipid phosphate phosphohydrolase 3-like**	***PPAP2B***	**Cell adhesion and cell-cell interactions**	**0.00637582**	**-4,17601**

Table 3.3.1: Genes downregulated in equine AMs after LPS stimulation

The Biolayout *Express*^{3D} software package was used to further analyse the gene expression of AMs in response to LPS stimulation. Data were normalised as described in **Section 2.10** and afterwards was clustered using correlation of r=0.91 and MCL of 2.2, generating a graph of 2,983 nodes connected with 690,178 edges that resulted in 22,778 clusters **(Figure 3.3.7.A)**. Analysis confirmed the results obtained by Partek Genomic Suite 6.6 software. LPS-regulated genes were found within several clusters (cluster 8, 11, 39, 62, 66) that followed a pattern similar to that of cluster 8 shown in **Figure 3.3.7.C**. Cluster 8 was the largest cluster of LPS-induced genes, consisting of 119 genes **(Figure 3.3.7C)**, while cluster 12 was the largest cluster of genes downregulated by LPS, consisting of 94 genes **(Figure 3.3.7 D)**, alongside clusters 20, 27, 52 and 59. These clusters included genes involved in RNA/DNA processes (DNA-directed RNA polymerase II subunit RPB4, tRNA-splicing ligase RtcB homolog, ribosomal RNA small subunit methyltransferase NEP1, methionyl-tRNA formyltransferase, mitochondrial), signal transduction (*LPXN*), apoptosis (*PTPN6*, *AEN*) and other cellular processes (*PLA2G15*, *SLC46A1*). A number of these genes were already detected previously in **Table 3.3.1**. The full genelists of the five larger clusters with genes that were up and downregulated in LPS treated equine AMs are presented in **Appendix III, S2** and **S3**, respectively.The top five clusters of the downregulated genes were explored for gene ontology by DAVID annotation software **(Table 3.3.2)** and included genes involved mainly in cellular metabolic processes and synthesis or assembly of the ribonucleoprotein complex.

Annotation cluster	Biological process	Enrichment score	Gene count
1	Ribonucleoprotein complex biogenesis	3.77	6-15
2	Nucleic acid transport	2.39	5-6
3	Cellular metabolic process	1.79	16-118
4	Mitochondrion organization / protein transport	1.2	3-33
5	Cellular component biogenesis	1.07	4-35
6	Ribonucleoside metabolic process	1.02	3-6

Table 3.3.2: The top six annotation clusters with downregulated genes.

The table shows the top 6 out of 40 annotation clusters created by DAVID software that were related with the list of 227 downregulated genes. The enrichment score is a modified Fisher exact p value calculated by the software and the higher it is the more enriched the cluster is. Each annotation cluster contains similar biological subcategories (http://david.abcc.ncifcrf.gov/content.jsp?file=functional_annotation.html). Each biological subcategory contains a specific number of genes; therefore, it is possible for a group of differentially expressed genes to be related to a particular annotation cluster via one or more of these biological sub categories. The range of gene counts (right column) incorporates the different gene counts relating to each biological subcategory.

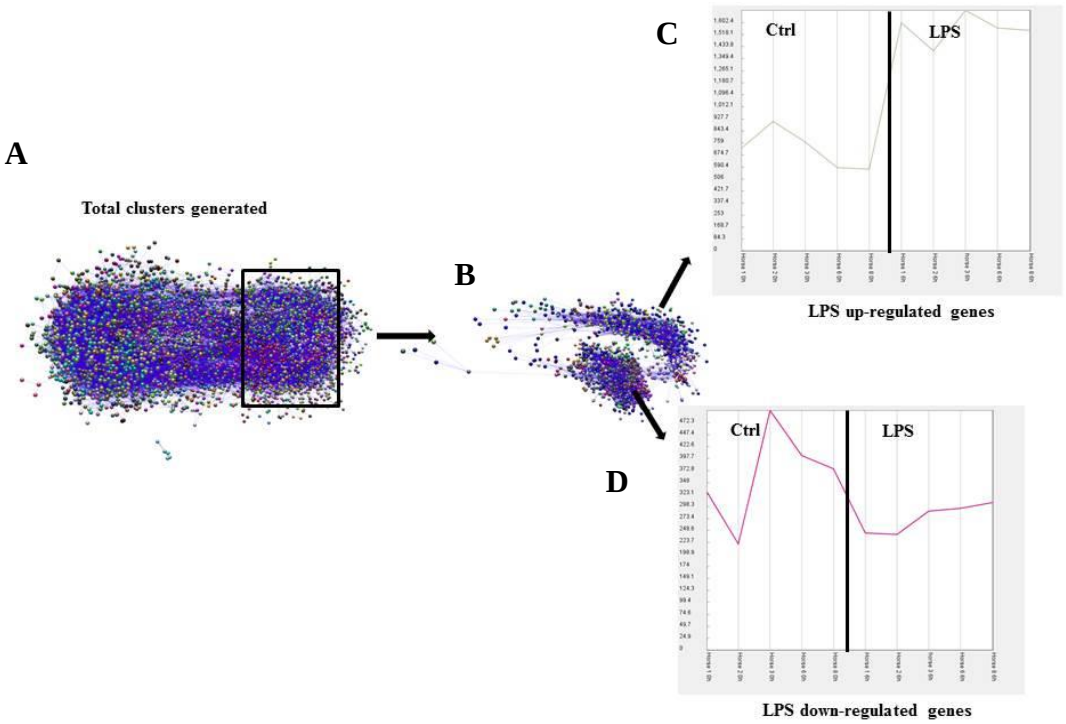


Figure 3.3.7: LPS regulated genes were grouped using Biolayout Express^{3D}.

*Genes were clustered by Biolayout Express^{3D} and graph **(A)** was generated. Of the LPS regulated genes **(B),** five were upregulated and five downregulated. Cluster 8 **(C)** is the biggest cluster of upregulated genes and cluster 12 **(D)** the biggest of those down-regulated. On the right panel the x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 1, 2, 3, 6 and 8, as depicted in order (left to right) on the x-axes were included in this analysis.*

Consistent with the Partek analysis, analysis of the genelist of the five largest LPS induced gene clusters (cluster 8, 11, 39, 62, 66), consisting of 319 genes in total, using the DAVID annotation tool, demonstrated enhanced expression of genes involved in immune system process, response to stimulus and positive regulation of cellular and biological process. These included *PTX3*, *GBP5*, *CCL5*, *IFIH1*, *TLR3*, as shown in **Figure 3.3.8.A-L**, in addition to others. As widely reported in the scientific literature, inflammation suppressor genes are also induced in response to LPS (Wells et al., 2005). Such genes include *IL1RN* and *SOCS3*, which were included in the LPS-induced gene list reported in the current study **(Figure 3.3.8.M-N)**.

The phenomenon of constant interaction between inflammation and apoptosis is well established and is vital for both homeostasis and the development of an immune response (Joshi et al., 2003). Consequently, the LPS-induced expression of many genes related to apoptosis and programmed cell death detected in the current study was consistent with this phenomenon. These included programmed cell death 1 ligand 1 (*PDCD1LG1*, also known as *CD274*, death-associated protein kinase 1(*DAPK1*), apoptosis regulator Bcl-2 (*BCL2L1*) and FADD-like apoptosis regulator (*CFLAR*), apoptotic peptidase activating factor 1(*APAF1*), various caspases (*CASP4*, *CASP7*, *CASP8*) and ubiquitin specific peptidase 37 (USP37) **(Figure 3.3.9.A-I)**. Numerous transcription factors, such as *STAT4*, ETS variant 6 (*ETV6*), *ETV7*, transcription factors GATA-6, transcription factor EC (*TFEC*) and basic leucine zipper transcriptional factor ATF like 3 (*BATF3*) were also found **(Figure 3.3.9.J-O)**.

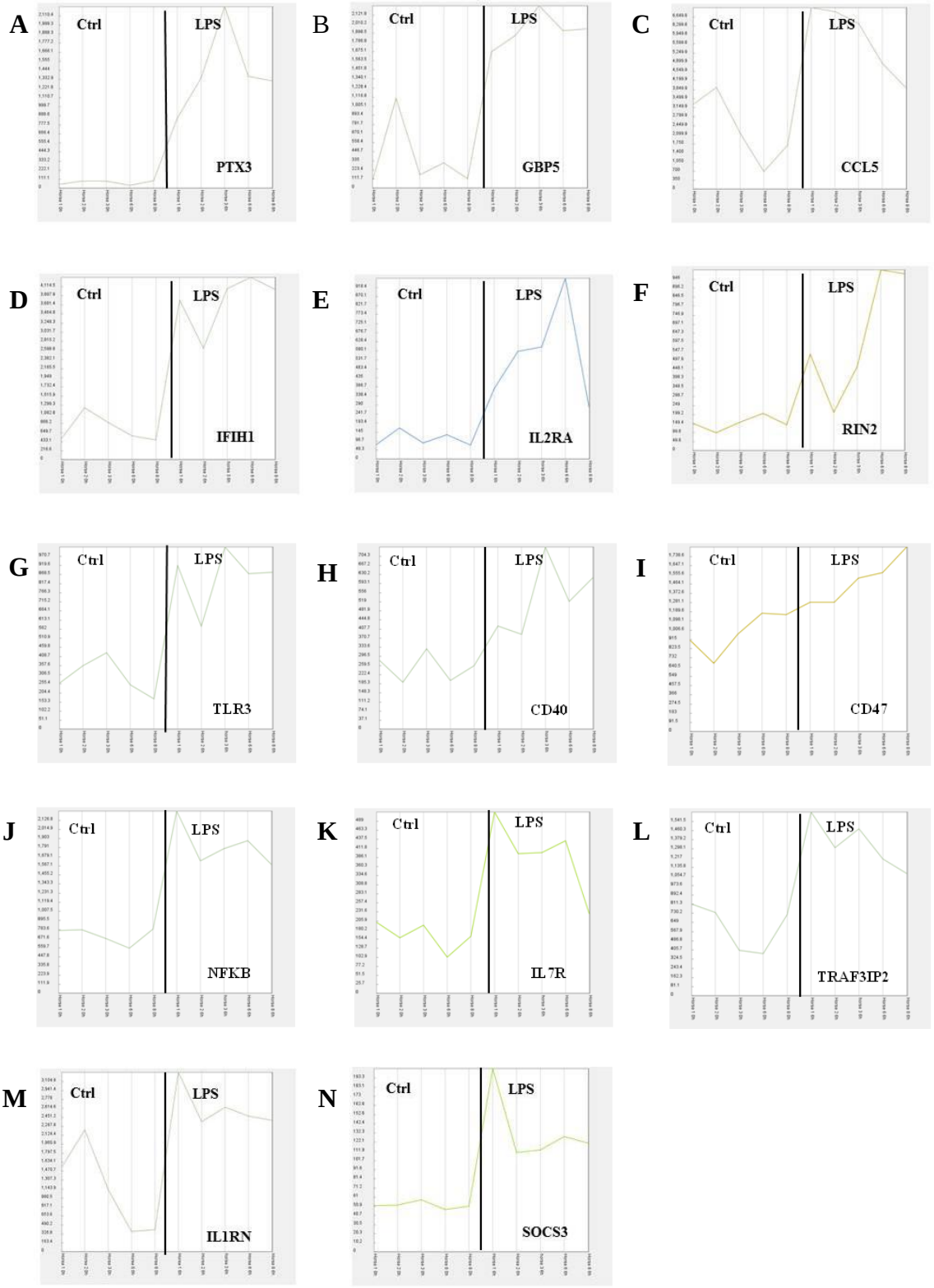


Figure 3.3.8: Expression profiles of positive (A-L) and negative regulators of immune response (M-N).

Selection of genes presented here were induced by LPS. These include genes which were located within the top 5 clusters of upregulated genes (e.g. genes 8, 11, 39 and 62). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 1, 2, 3, 6 and 8, as depicted in order (left to right) on the x-axes were included in this analysis.

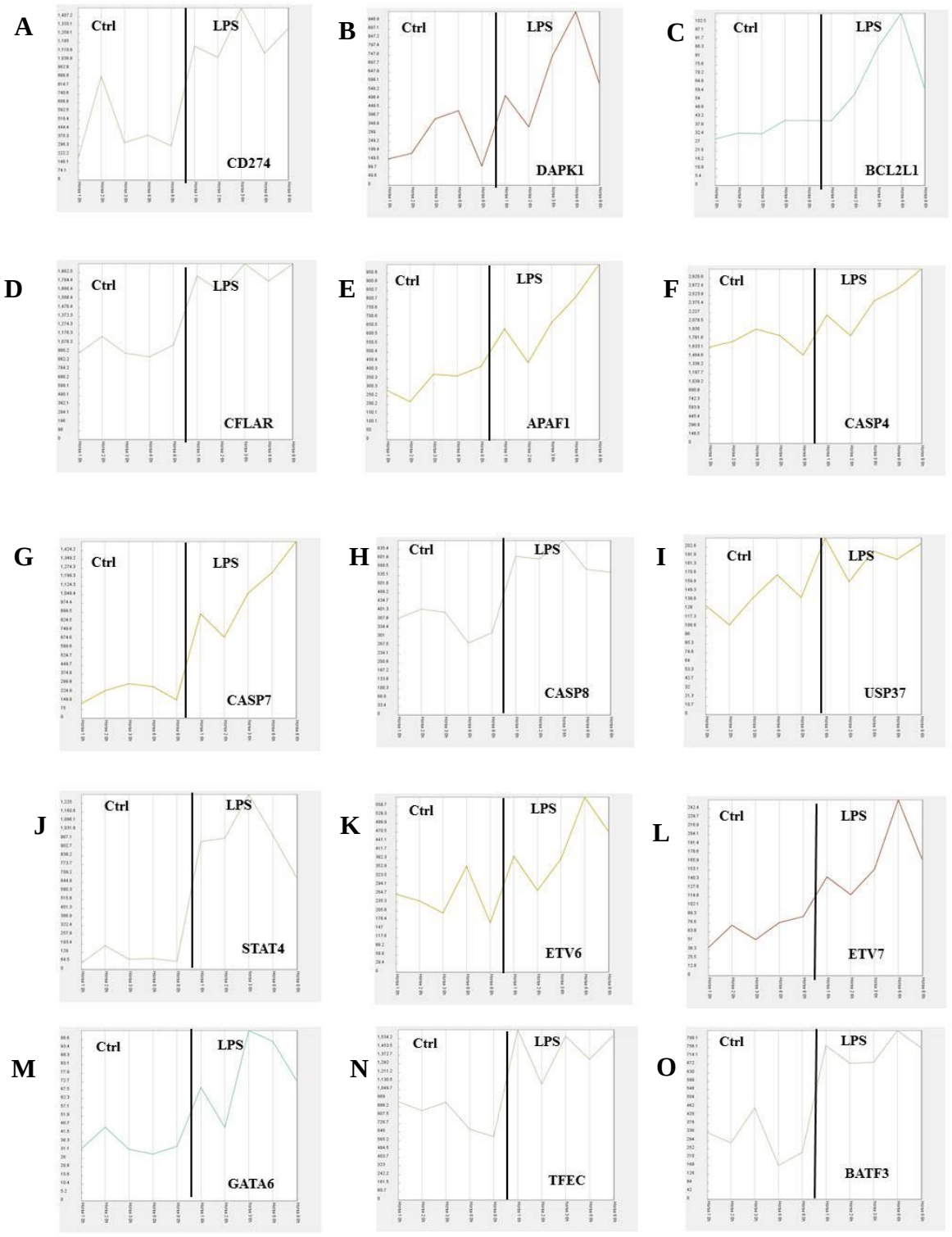


Figure 3.3.9: Expression profiles of genes associated with apoptosis-cell death (A-I) and of transcription factors (J-O).

Selection of genes presented here were induced by LPS These include genes which were either (a) located within the top 5 clusters of upregulatedgenes (e.g. genes 8, 11, 66) or (b) located within

other smaller clusters, yet with a similar expression profile and considered to have potential biological significance (e.g TFEC and BATF). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 1, 2, 3, 6 and 8 were included in this analysis, as depicted in order (left to right) on the x-axes.

A large number of genes responsible for the initiation and the maintenance of inflammation were detected among the five largest clusters and other clusters with a similar expression profile; for example members of the TNF and TNF receptor superfamily, (*TNF*, *CD40*, *TNFSF13B*), interleukins (*IL1α*, *IL1β*, *IL6*) and chemoattractant chemokines (*CCL2*, *CCR5*, *CXCL1*, *CXCL6*, *CXCL9*) (Gaur and Aggarwal, 2003). Moreover, *IL7R*, which plays a critical role in lymphocyte development, and the positive regulator of Th1 type cytokine expression, PHD finger protein 11 (*PHF11*) were also upregulated after LPS stimulation. The inducible gene list also included several secreted growth factors or their receptors, including Schlafen family member 5 (*SLFN5*), *TGFB1*, *GM-CSF*, colony stimulating factor 2 receptor beta (*CSF2RB*) and *CSF3*. Interestingly, even though IL10 protein was not detected in the cell culture supernatant at 6h and 24h follow LPS stimulation of AMs, *IL10* gene expression was induced at 6h post LPS stimulation. Finally, *BANK1* expressed on B cells, *CCL1*, *CD2*, *CD7* on T cells and *CD5* expressed by both were also identified in different clusters, indicating some lymphocyte contamination, a phenomenon also observed in previous studies (Kapetanovic et al., 2012, Waldschmidt et al., 2013).

3.3.1 Comparative analysis with human data sets

Humans and horses are more sensitive to endotoxaemia compared to other species such as mice. Several studies have identified significant differences between human and murine responses to LPS which likely result from species-specific differences in pathophysiological characteristics (Fairbairn et al., 2011, Schneemann et al., 1993, Schneemann and Schoeden, 2007, Junhee et al., 2013, Schroder et al., 2012). For example, the difference in cell responses to bacterial infection has been partly

attributed to species-specific differences in serum proteins (Warren et al., 2010). LPS stimulation of macrophages (including AMs) incubated with mouse serum resulted in a lower expression of endotoxin induced proteins compared to cells incubated with human serum (Warren et al., 2010). As a result, it has been suggested that the horse may be an more appropriate animal model for human endotoxaemia compared to the widely used rodents. In order to address this hypothesis, a comparison was made between horse and human LPS treated AMs.

LPS stimulated AMs have been profiled in several species, including humans. Reynier and colleagues performed microarray analysis on human AMs harvested from seven individuals 6h following LPS (4 ng/kg body weight) instillation into a lung subsegment (Reynier et al., 2012). Control samples were retrieved from the contralateral lung of the same subjects 6h following saline instillation (Reynier et al., 2012). As noted above, horse macrophages expressed several genes (*IDO*, *IL7R*) that are shared with humans and pigs, but not mice. There is increasing demand for the development of novel animal models which more closely resemble human pathophysiology compared to mice (for example pigs). To assess whether the horse might be a good model for human inflammation, the published human microarray datasets derived from the Reynier *et al* (2012) study (GDS4419) **(Section 2.10.1)** were compared with those derived from the horse in the current study.

The human arrays were normalised and analysed as described above for the horse, using Partek Genomic Suite 6.6 software. The PCA showed a distinct separation between the LPS treated and untreated AMs **(Figure 3.3.10)**. In agreement with the equine AM-derived microarray data (**Figure 3.3.2.B**), the source of variance plot revealed "treatment" (LPS) and "human subject" to be the factors which had the greatest effect on gene expression **(Figure 3.3.11)**.

Using a two way ANOVA ($p<0.05$) and fold change of two or more, a gene list of 1,085 genes that were statistically significantly different between the two human AM groups was created. A total of 966 out of 1,085 genes were upregulated and the remainder (118 genes) downregulated following LPS stimulation **(Appendix III, S4)**. The upregulated genes included many proinflammatory genes (e.g. *IL6*, *IL8*,

IL1β, *IL1RA*), some of which were also upregulated in horse AMs in the current study following *ex vivo* LPS stimulation **(Figure 3.3.5)**. The hierarchical clustering of the genelist demonstrated that gene expression between LPS and saline treatment (control) was clearly different **(Figure 3.3.12)**.

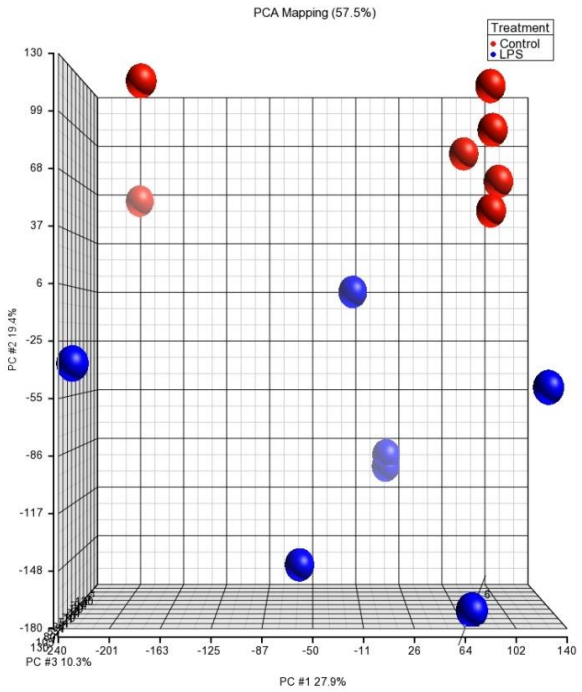


Figure 3.3.10: Principal component analysis of LPS treated and untreated human AMs.

After 6h in vivo challenge of human AMs with LPS (4 ng/kg body weight) or saline, cells were isolated from BALF, RNA was extracted and microarray analysis was performed. This is the PCA of LPS treated and saline treated (control) human AMs. Blue spheres represent the LPS treated AMs and red spheres represent saline treated AMs (controls). The relative difference in colour intensity for each group reflects the fact that a 3-dimensional image has been transcribed onto a 2-dimensional figure. Analysis was performed by Partek Genomic Suite 6.6 software. This is a three dimensional presentation of the microarray experssion data. The proportions in each axis (PC#1, PC#2 and PC#3) represent the percentage of variance explained by each component. The percentage on the top of the graph (57.5%) represents the sum of these percentages.

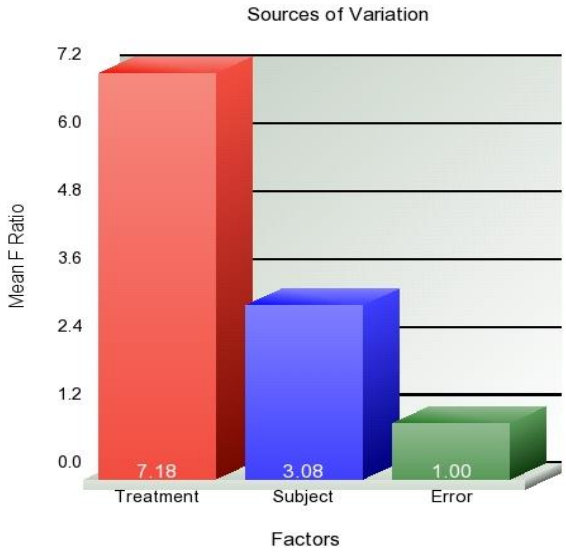


Figure 3.3.11: Sources of variation plot.

View of plots by Partek Genomic Suite 6.6. The histogram presents the average effect size across all the genes for each of the factors (x-axis). The error column represents the noise or the variability of the data not explained by the rest of the factors and the subject column represents the seven different human volunteers that took part on this experiment. The y-axis represents the mean F Ratio (signal-to- noise) of all the genes.

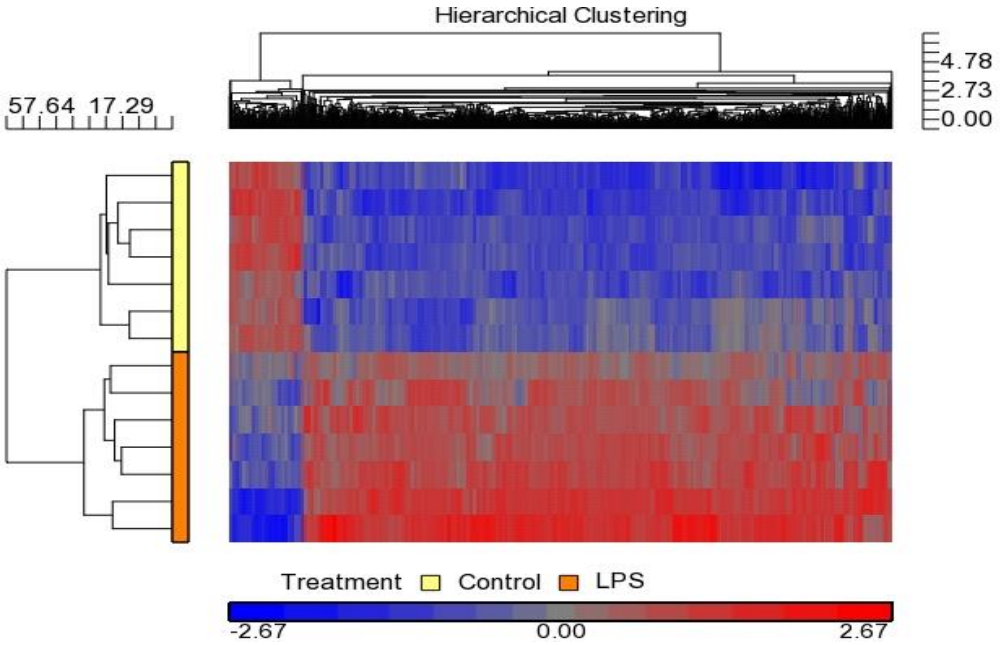


Figure 3.3.12: The hierarchical cluster diagram of gene expression profiles in LPS/saline treated AMs of seven individuals.

The expression patterns between saline-control (yellow dendrogram) and LPS (orange dendrogram) samples. Each column shows the expression of 1,085 probe sets expressed by each sample. Red indicates genes that were upregulated and red those that were downregulated.

Despite the fact that the annotation of the horse genome is not yet complete, the analysis revealed 66 orthologous genes between the horse and human total genelists. Comparison between horse and human AMs in response to LPS **(Figure 3.3.13)** revealed a marked similarity in gene expression, a finding which may partly explain the similarities between horses and humans with regard to their susceptibility to endotoxaemia (Moore and Barton, 1998, Kobayashi et al., 2013).

Previous work has identified LPS-induced genes shared by human and porcine, but not murine, monocyte-derived macrophages (Kapetanovic et al., 2012, Kapetanovic et al., 2013, Schroder et al., 2012). Many of these were also induced in LPS treated equine AMs in the current study. These comprised genes already discussed above (*STAT4*, *IDO1*, *IL7R*, and *BATF3*), as well as G-protein coupled receptor 183 (*GPR183;* involved in B cell activation and proliferation), *CD80* (involved in T cell activation and proliferation), RNA binding motif, single stranded interacting protein 2 (*RBMS2*), Rap guanine nucleotide exchange factor 2 (*RAPGEF2*), poly (ADP-ribose) polymerase family, member 9 (*PARP9*); kynureninase (*KYNU;* involved in the IDO pathway) and the apoptosis inhibitor XIAP associated factor 1 (*XAF1*) **(Figure 3.3.14)**.

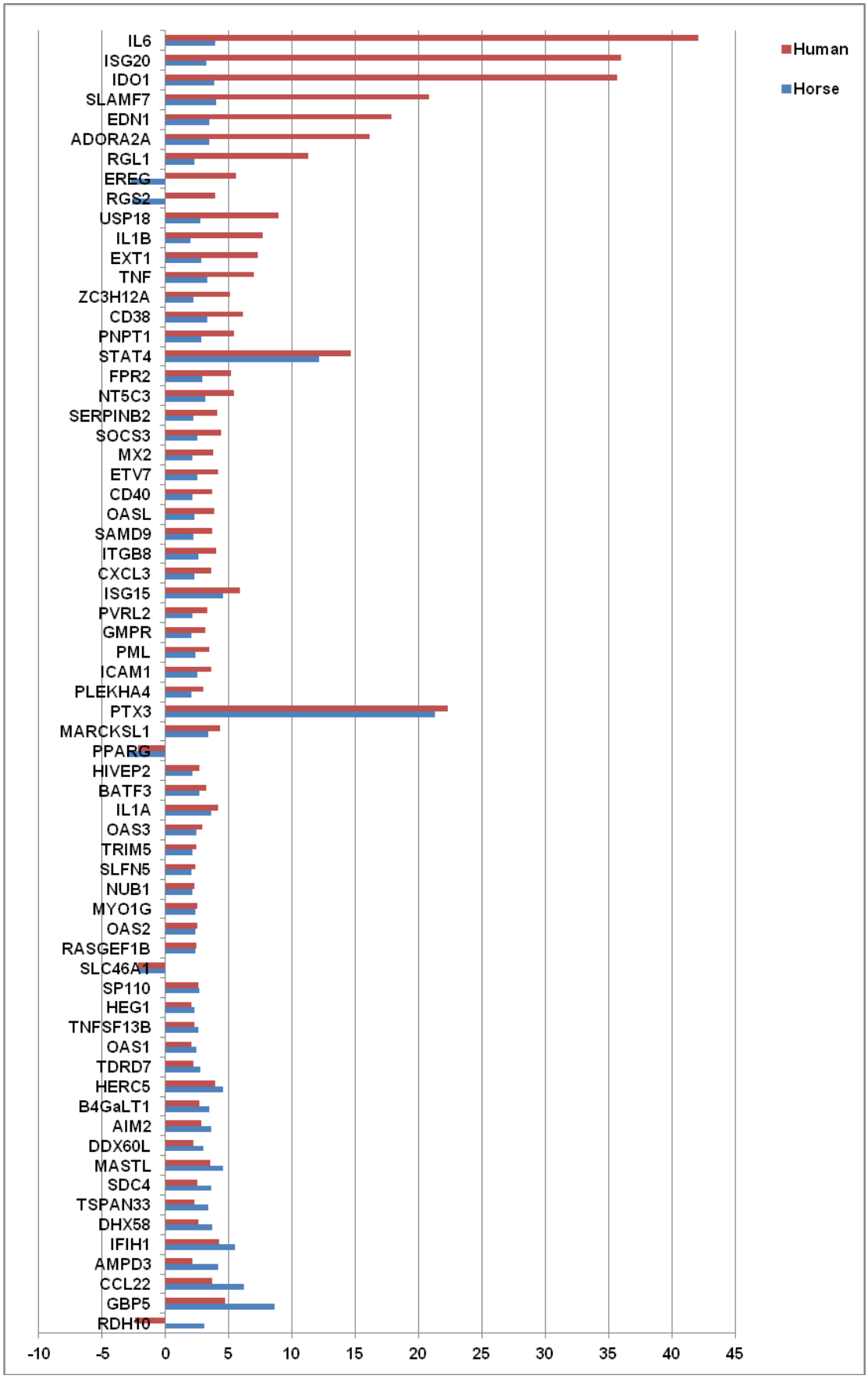

Human
Horse
IL6
ISG20
IDO1
SLAMF7
EDN1
ADORA2A
RGL1
EREG
RGS2
USP18
IL1B
EXT1
TNF
ZC3H12A
CD38
PNPT1
STAT4
FPR2
NT5C3
SERPINB2
SOCS3
MX2
ETV7
CD40
OASL
SAMD9
ITGB8
CXCL3
ISG15
PVRL2
GMPR
PML
ICAM1
PLEKHA4
PTX3
MARCKSL1
PPARG
HIVEP2
BATF3
IL1A
OAS3
TRIM5
SLFN5
NUB1
MYO1G
OAS2
RASGEF1B
SLC46A1
SP110
HEG1
TNFSF13B
OAS1
TDRD7
HERC5
B4GaLT1
AIM2
DDX60L
MASTL
SDC4
TSPAN33
DHX58
IFIH1
AMPD3
CCL22
GBP5
RDH10
-10
-5
0
5
10
15
20
25
30
35
40
45

Figure 3.3.13: The horse AM response to LPS was similar to that of human AMs.

Fold change of gene expression between horse (current study) and human (GDS4419) microarray data derived from LPS treated and control AMs. Genes with fold change greater than 2, which were statistically significantly different between the LPS treated AMs and controls were included in the comparison.

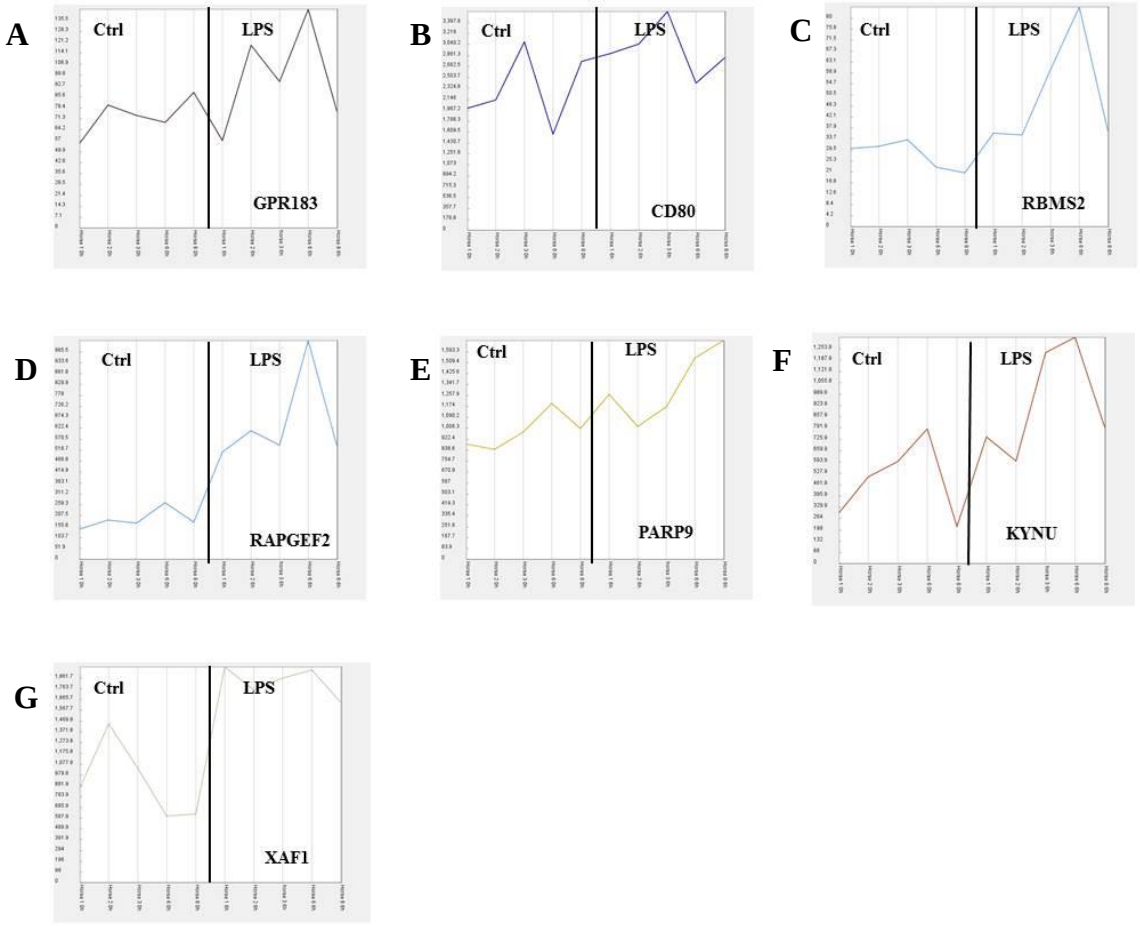


Figure 3.3.14: Expression profile of genes upregulated in LPS stimulated equine macrophages.

These genes are shown to be also upregulated in human but not in mouse LPS treated macrophages. These include genes which were either (a) located within the top 5 clusters of upregulatedgenes (e.g. 11 and 62 or (b) located within other smaller clusters, yet with a similar expression profile and considered to have potential biological significance (e.g GRPI83, CD80, KYNU and XAF1). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse. Horses 1, 2, 3, 6 and 8 were included in this analysis, as depicted in order (left to right) on the x-axes.

Transcripts for genes important for the NO pathway, such as GTP cyclohydrolase 1 (*GCH1*), *iNOS*, argininosuccinate synthase 1 (*ASS1*), arginase 2 (ARG2), ornithine aminotransferase (*OAT*), argininosuccinate lyase (*ASL*), were included in the Affymetrix equine microarray strip. However they were not induced by LPS stimulation, further emphasising the distinctions between rodents and humans, horses and pigs **(Appendix III, S1 and S2)**. RT qPCR was also used to validate the equine microarray data regarding gene expression of *IL7R* , *STAT4* and (TNFAIP3-interacting protein 3) *TNIP3* **(Figure 3.3.15)**, as well as of *TNFα* and *IDO*, which were verified earlier **(Figure 3.2.4)**. Their microarray gene expression is shown in **Figure 3.3.16**.

The vitamin D_3 pathway is shown to play an important role in protection against infections (Gombart, 2009) and the *CYP27B1* gene, involved in this pathway, is induced by LPS in both porcine and human macrophages, but not in murine macrophages (Kapetanovic et al., 2012, Kapetanovic et al., 2013, Reynier et al., 2012). In the current study on equine AMs, LPS-induced expression of this gene was very low (microarray fluorescence intensity <50 and dynamic range after LPS treatment <1.5), a finding verified by subsequent RT qPCR analysis (Ct values > 30, *data not shown*). Interestingly, the circulating concentration of vitamin D in horses is very low, and does not appear to play a key role in regulating calcium and inorganic phosphate [P(i)] in homeostasis (Breidenbach et al., 1998). Thus, this may be a species-specific trait.

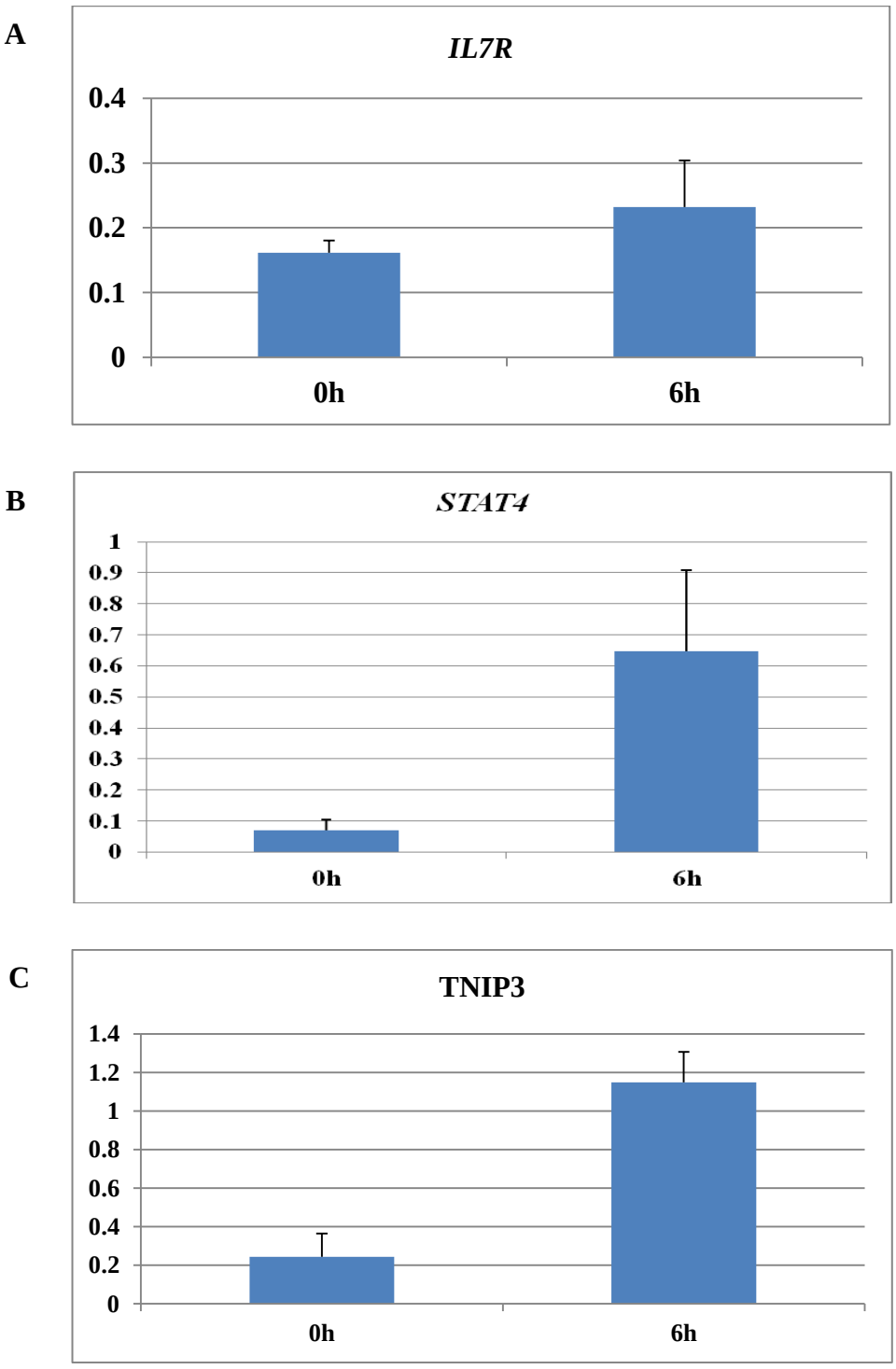


Figure 3.3.15: LPS induced IL7R (A), STAT4 (B) and TNIP3 (C) in equine AMs.

Horse AMs were treated with LPS and incubated for 6h. RNA was extracted from the same samples used for the microarrays and reverse transcribed. mRNAs for IL7R ***(A)****, STAT4****(B)*** *and TNIP3* ***(C)*** *were measured by RT qPCR. Results are representative of 5 experiments +/-SEM, using RNA from 5 different animals (horse 1, horse 2, horse 3, horse 6, horse 8).*

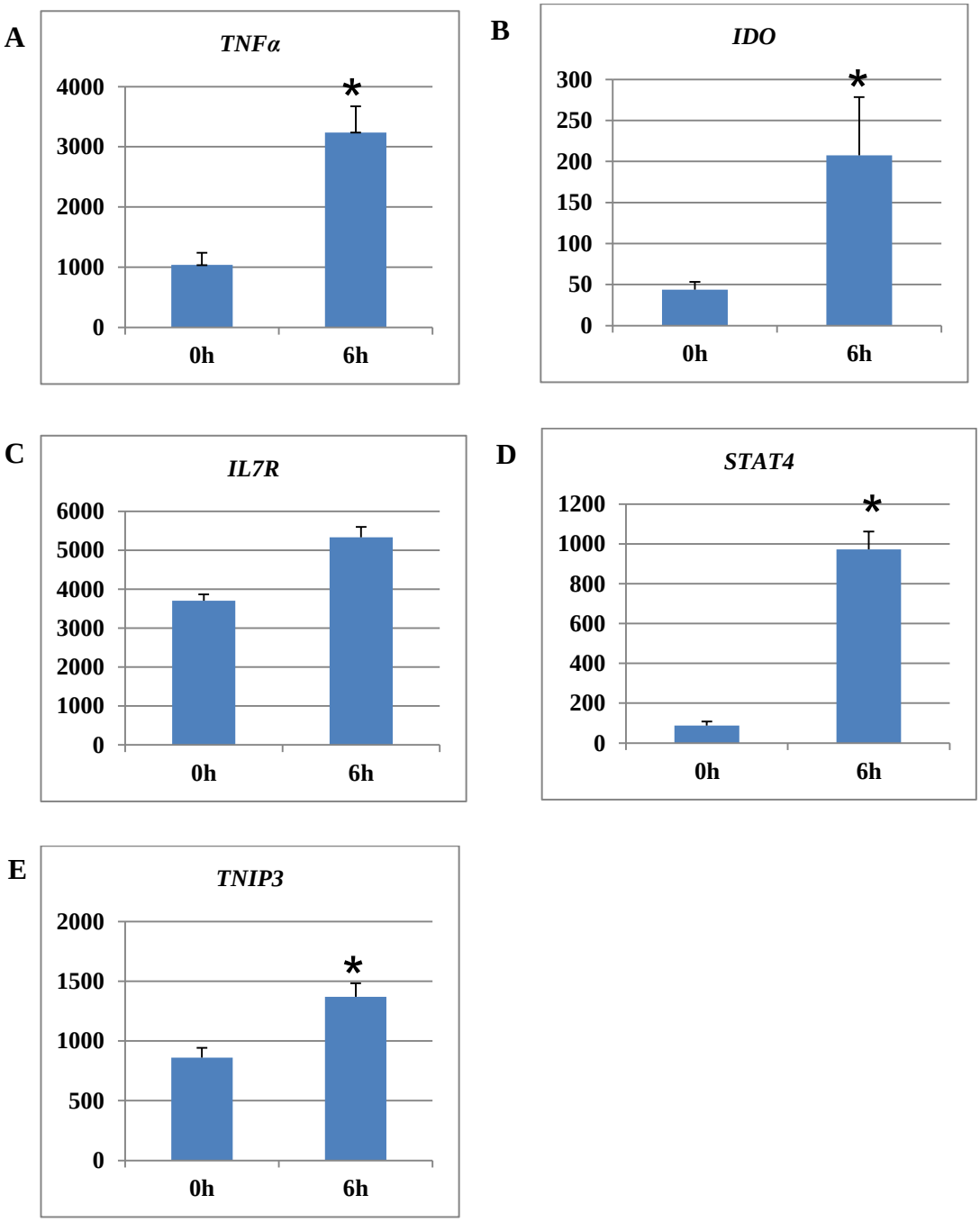


Figure 3.3.16: The mean expression of (A) TNFα, (B) IDO, (C) IL7R, (D) STAT4 and (E) TNIP3 probesets as estimated during the microarray analysis on LPS treated (6h) and untreated (0h) equine AMs.

Results are representative mean of 5 experiments +/-SEM, using RNA from 5 different animals (horse 1, horse 2, horse 3, horse 6 and horse 8). Statistical significance (*) was set at $p<0.05$ versus control (0h). IL7R induction was not statistically significantly different from control, however its induction

by LPS was detected in Biolayout cluster 11 as previously shown in ***Appendix III S2*** *and* ***Figure 3.3.8***.

3.4 The effect of race training on the basal gene expression of AMs derived from Standardbred racehorses

Several human studies have focused on the effect of exercise on circulating monocytes, with respect to numbers, population shifts, cell surface receptor expression and cytokine production (Okutsu et al., 2008, Krueger and Mooren, 2007, Lancaster et al., 2005, Hong and Mills, 2008, Starkie et al., 2001, Steppich et al., 2000). Similarly, more limited studies in horses have utilized microarray technology to assess the effects of long duration, extended exercise on peripheral blood monocyte global gene expression (Barrey et al., 2006, Capomaccio et al., 2010). Although such work has provided valuable information on exercise-induced alterations in immunity, it is limited with respect to offering insights into exercise-induced and/or training-associated changes in resident macrophage function and related immune responses at the tissue level (Walsh et al., 2011). Some studies investigating the effect of exercise on AMs have been conducted in humans (Fehr et al., 1989), mice (Ceddia et al., 2000, Woods et al., 1997, Kohut et al., 1998) and horses (Raidal et al., 2000) and have demonstrated an exercise-associated reduction in MHC-II expression (Woods et al., 1997), antigen presentation capacity (Ceddia et al., 2000), phagocytic properties (Raidal et al., 2000) and impaired response to several stimuli (Kohut et al., 1998, Mignot et al., 2012). To date, no studies have investigated alterations in the overall gene expression profile of equine AMs in response to extended training.

Accordingly, this section of work aimed to define the gene expression of AMs derived from eight standardbred racehorses prior to and following commencement of race training. BALF from eight systemically healthy Standardbred racehorses was collected at two different time points: prior to entry into training (T0) and during the training period (T1). Inclusion criteria for T1 sampling included the requirement for the horses to have been in training for at least 10 days prior to sample collection.

3.4.1 Cell recovery and populations

Cell viability on the day of cell harvesting was greater than 80%. Total cell counts [200(±23)/mm^3 for T0 and 255(±43)/mm^3 for T1] were not statistically significant different between the two time points, although slightly increased during the training period. Differential cell counts of raw BALF were assessed and are shown in **Tables 3.4.1** and **3.4.2**. A slightly greater neutrophil percentage was found in samples obtained from horses during the training period, as reported previously (McKane et al., 1993). The mean percent of haemosiderophages was also increased during the training period, consistent potentially with a previous episode of EIPH, a disorder commonly observed in racehorses undergoing training (Doucet and Viel, 2002). However, no statistical statistical significance was observed on these results.

According to the formal definition of IAD (Robinson, 2003, Couetil et al., 2007), all horses from T0 apart from BU11 and all from T1 were affected with the disease. Since no clinical symptoms were observed on the horses in this study, the form of the disease was considered as subclinical. Following overnight culture and removal of nonadherent cells, more than 90% of adherent cells were identified morphologically as macrophages, by light microscopy.

DCCs (%) (T0), n=7	**BU1**	**BU2**	**BU4**	**BU5**	**BU6**	**BU11**	**BU12**	***Mean+/-SEM***
Macrophages	42.5	21.5	44	66	45	60	57.5	***48.1+/-5.6***
Lymphocytes	32	26.5	39	23.5	27.5	34	34.5	***31+/-2***
Haemosiderophages	5.5	29	-	1.5	9	1	-	***6.6+/-3.9***
Neutrophils	11	5	12	5	5	4	2	***6.3+/-1.4***
Eosinophils	-	15.5	2	-	12	-	-	***4.2+/-2.5***
Mast cells	9	2.5	2	4	1.5	1	5.5	***3.6+/-1.2***
Multinucleated giant cells and/or basophils	-	-	1	-	-	-	0.5	***0.2+/-0.2***

Table 3.4.1: Differential cell count (%) of BALF derived from Standardbred horses prior to entry into race training (T0).

DCCs (%) (T1), n=8	BU1	BU2	BU4	BU5	BU6	BU8	BU11	BU12	*Mean+/-SEM*
Macrophages	50	14	47	64	51	22	57	61,5	***45.8+/-6.4***
Lymphocytes	31	19	33	30	19	31.5	35.5	27.5	***28.3+/-2.2***
Haemosiderophages	-	54	10.5	-	7	29,5	-	1	***12.8+/-6.9***
Neutrophils	16.5	7	7.5	3.5	16.5	16	5.5	6	***9.8+/-2***
Eosinophils	0.5	-	-	-	-	-	0.5	-	***0.2+/-0.1***
Mast cells	1.5	6	1.5	2.5	6.5	1	1.5	3.5	***3+/-0.8***
Multinucleated giant cells and/or basophils	0.5	-	0.5	-	-	-	-	0.5	***0.2+/-0.1***

Table 3.4.2: Differential cell count (%) of BALF derived from Standardbred horses following initiation into (T1) race training.

3.4.2 The effect of race training on TNFα and IL10 production by AMs derived from Standardbred racehorses

As noted above, some previous studies suggest that intense exercise might affect the function of AMs (Wong et al., 1990, Raidal et al., 2000, Raidal et al., 2001). For example Raidal and colleagues found that strenuous exercise was associated with impaired phagocytosis and increased oxidative burst activity of AMs (Raidal et al., 2001, Raidal et al., 2000). Exposure of macrophages to low levels of LPS can produce prolonged refractoriness to restimulation **(Section 3.1.8)**. Hence, a possible indicator of exposure *in vivo* to an LPS-like microbial stimulus during exercise would be a change in LPS-responsiveness *ex vivo*. To address this possibility, TNFα in AM culture supernatant was measured by ELISA before and 6h following LPS stimulation. The levels of basal TNFα production (0h) induced in AMs from racehorses were similar to those observed previously **(Section 3.1.5)**. However, LPS-induced TNFα production by AMs derived from racehorses during the training period (T1) was slightly greater than that at T0 **(Figure 3.4.1)**. Consistent with previous results **(Section 3.1.5)**, LPS treatment did not induce detectable IL10 **(Figure 3.4.2)**.

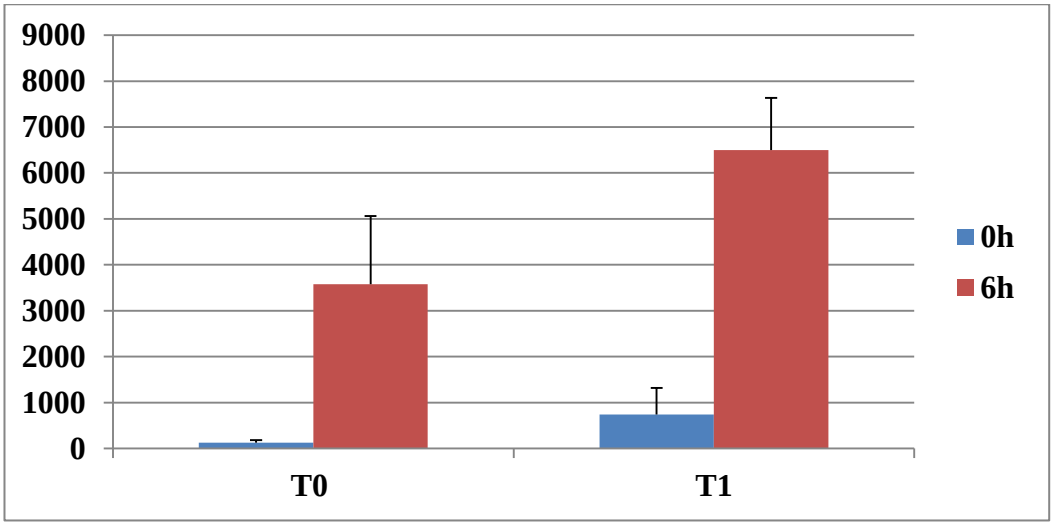


Figure 3.4.1: Supernatant TNFα concentration (pg/ml) in LPS treated AMs harvested from Standardbred racehorses prior to (T0) and following (T1) entry into race training.

BALF from Standardbred racehorses was collected before and during the training period. After AM isolation, cells were seeded in plastic plates at 1x10^6 cells and left overnight to adhere to the plastic. Next morning they were stimulated with 100ng/ml LPS for 6h, supernatant was collected and TNFα production was measured by ELISA. Results are the mean of a minimum of two experiments derived from different horses+/- SEM.

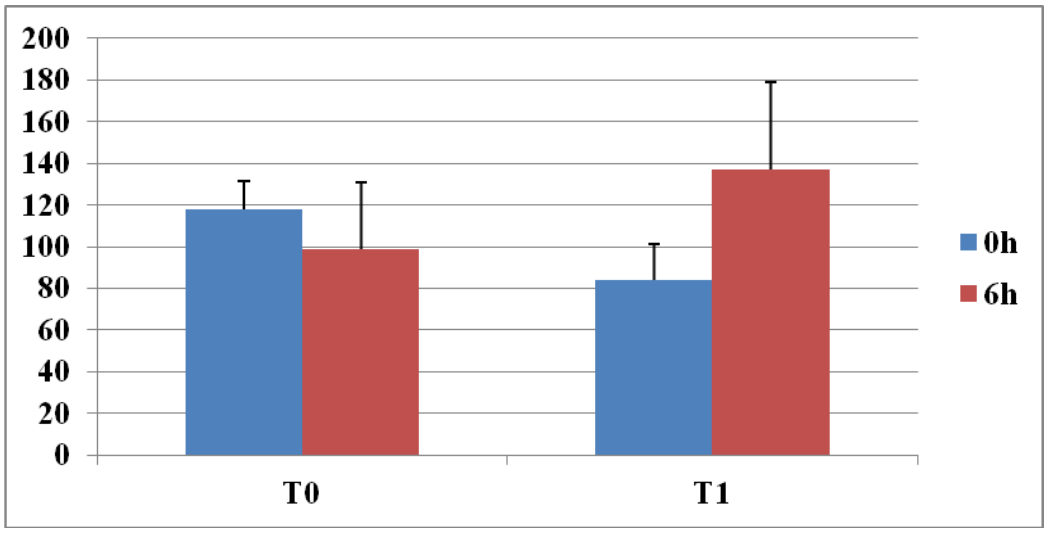


Figure 3.4.2: Supernatant IL10 concentration (pg/ml) in LPS treated AMs harvested from Standardbred racehorses prior to (T0) and following (T1) entry into race training.

Cells were treated as described previously ***(Figure3.4.1).*** *Results are the mean of a minimum of three experiments +/- SEM.*

3.4.3 Transcriptomic analysis of equine AMs before and during the training period

BALF was collected from eight Standardbred racehorses before and after the commencement of training as described in **Section 2.1.2**. After isolation of AMs by adherence, RNA was extracted at 0h and 6h following LPS (100ng/ml) stimulation, providing an average yield of 1.7µg of RNA /10^6 cells (± 0.5). Untreated RNA samples from 0h time point and with RNA integrity number (RIN) greater than 7 were used for microarray analysis (**Figure 3.4.3**). According to these criteria, RNA samples derived only from horse BU1, BU2, BU4, BU5, BU6 and BU12 (n=6) from the **Table 2.2** were used for the microarray screening.

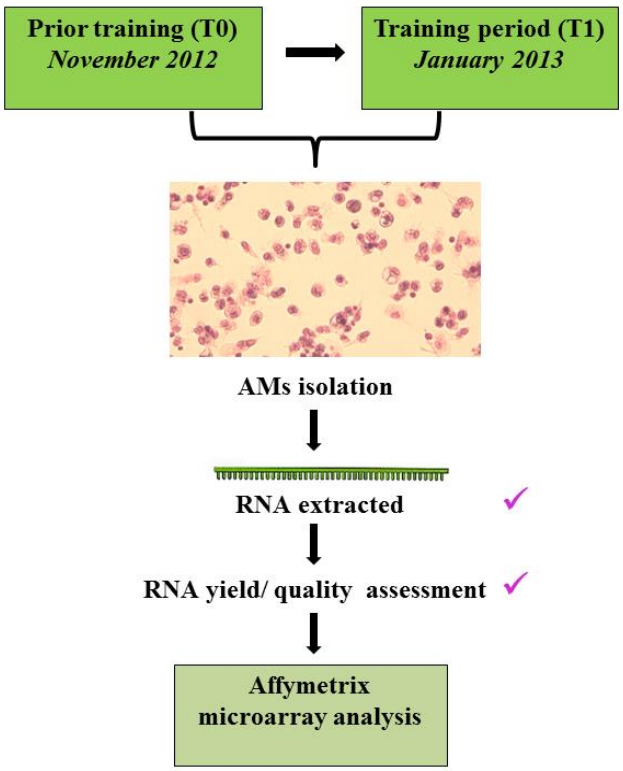
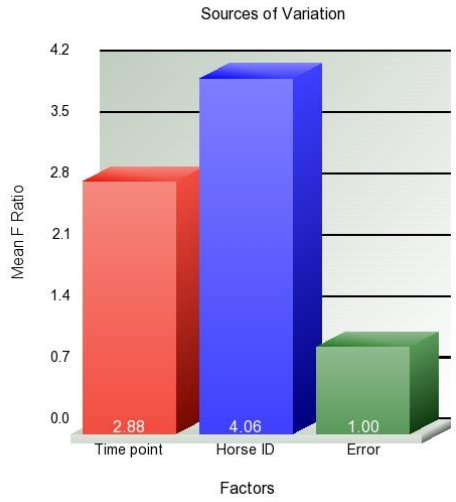


Figure 3.4.3: Workflow for studying the gene expression response of racehorse AMs to training.

BALF samples were collected at 2 different time points: prior the training period (T0, n=7) and during the training period (T1, n=8). Retrieved fluid was processed and isolated BALF cells were incubated overnight. Next morning RNA was extracted. After RNA quality assessment samples were used for microarray analysis (n=6). Cell figure captured from the current study.

All expression data were normalised in the Partek Genomics Suite 6.6 software and analysis was performed as previously described **(Section 2.9** and **3.2.6)**. The source of variation plot **(Figure 3.4.4.A)** depicts the average effect size of all factors included in the experiment. Removal of the horse ID as a batch effect revealed that training had a significant effect **(Figure 3.4.4.B)**. Nevertheless, the largest variation detected was between horses, an observation also confirmed by the PCA **(Figure 3.4.5)**. Unsupervised exploration of gene expression by PCA displayed a separation of samples into two groups corresponding to the pre-training period (T0) and the intra-training period (T1) **(Figure 3.4.5)**. Horses BU2, BU4 and BU6 were more tightly clustered together compared to the rest. Based on the information available on these horses (e.g. stage of training, gender), no common factor could be attributed to this grouping, with the exception of the fact that these horses represented the oldest in the group of 6 **(Table 2.2)**. However, no definitive conclusions could be drawn from the available information and further work on a larger dataset would be required to definitely address this question. A two-way ANOVA test (time point: fixed effect; horse ID: random effect) was performed in order to identify differentially expressed genes between the two groups. No statistically significant difference was observed between the two time points (T0 and T1) ($p < 0.05$, Fold change ≥ 2), potentially due to the relatively small sample sizes.

A



B

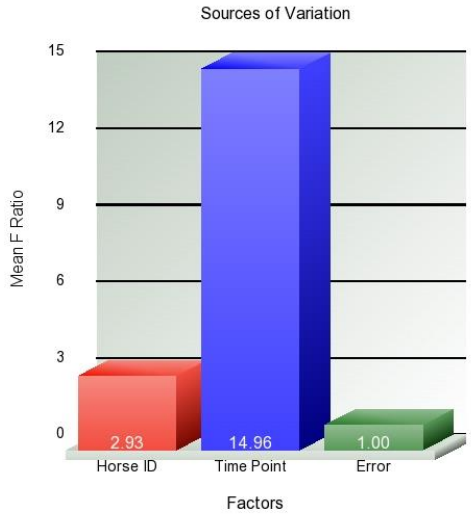


Figure 3.4.4: Source of variation plot of AMs from 6 racehorses before and during the training period.

View of plots by Partek Genomics Suite 6.6 software before ***(A)*** *and after* ***(B)*** *the horse ID was removed as a batch effect. These plots present the average effect size across all the genes for each of the factors. The y- axis represents the Mean F-ratio (signal-to-noise) of all the genes, while the x-axis represents the factors influenced the gene expression. The error column represents the noise or the variability of the data not explained by the rest of the factors.*

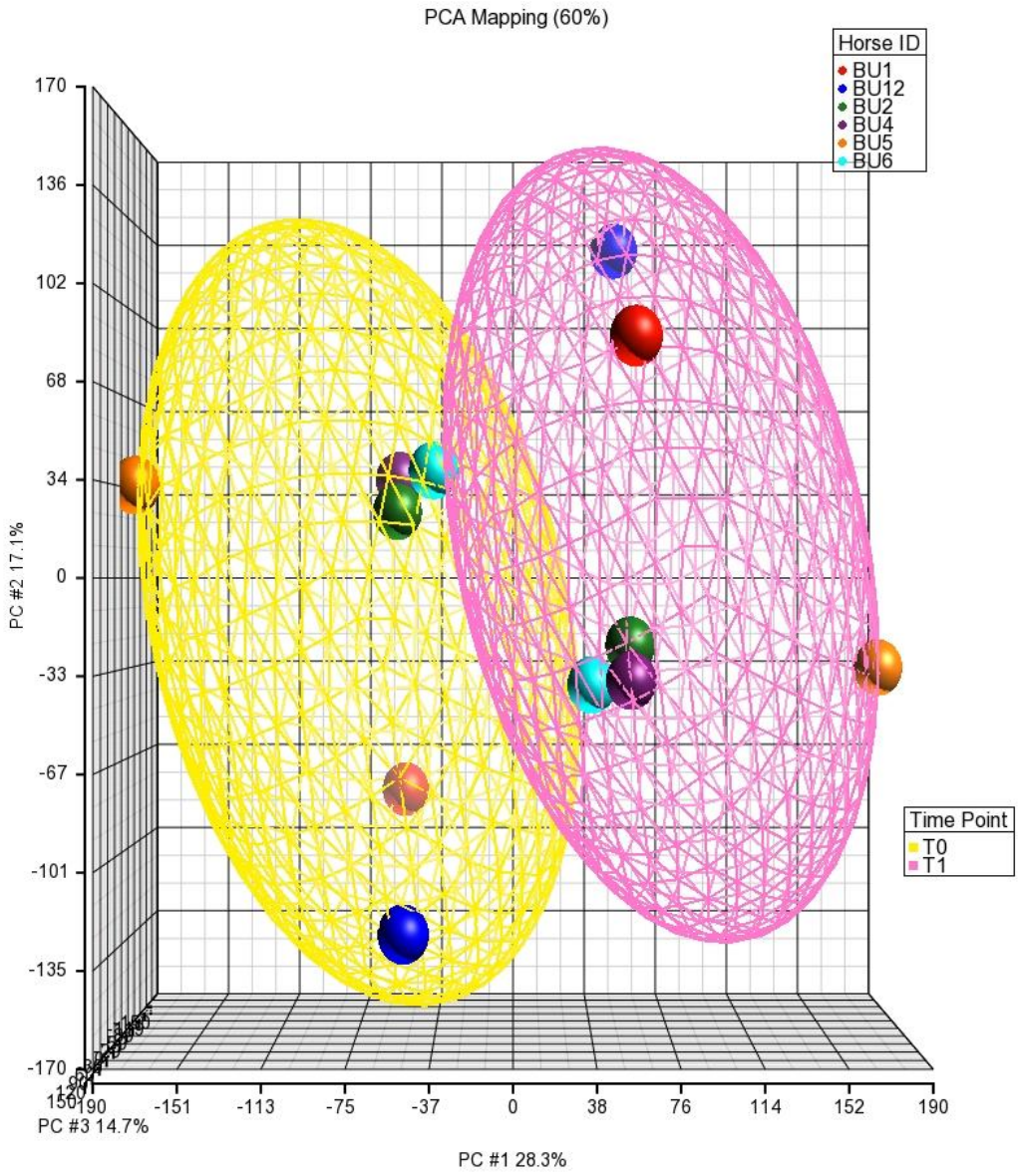


Figure 3.4.5: PCA of AMs from 6 racehorses before and during the training period.

Yellow and pink ellipsoid-webs represent the AMs retrieved while the racehorses were at rest (T0) and during the training period (T1), respectively. Each individual is represented with a different coloured sphere. The relative difference in colour intensity for each group reflects the fact that a 3-dimensional image has been transcribed onto a 2-dimensional figure. Analysis was performed by Partek Genomic Suite 6.6 software. This is a three dimensional presentation of the microarray experssion data. The proportions in each axis (PC#1, PC#2 and PC#3) represent the percentage of variance explained by each component. The percentage on the top of the graph (60%) represents the sum of these percentages.

Network analysis of the normalised expression data was conducted using Biolayout *Express*^{3D}. Only transcripts that were differentially expressed by dynamic range ≥ 1.5 were included in the analysis. As discussed earlier, Biolayout *Express*^{3D} is a software package used for the analysis of microarray data that allows detection of groups of genes with a similar expression profile (Freeman et al., 2007). Using this tool, a Pearson correlation matrix (r≥0.9) of a transcript-to-transcript comparison was used to filter for correlated expression patterns across the samples. This created a graph of 12,860 nodes connected by 104,012 edges and 4,117 clusters . The nodes correspond to transcripts (microarray gene probes) and the edges indicate a correlation between them above the threshold (r≥0.9). The clustering algorithm MCL with an inflation value of 2.2 was used to identify groups of tightly co-expressed genes and create clusters containing a minimum of three nodes (van Dongen and Abreu-Goodger, 2012).

Figure 3.4.6 shows the network graph of all the genes based on co-expression patterns (r ≥ 0.9, MCL inflation: 2.2), in which clusters of genes with greater expression at T0 and clusters of genes with greater expression at T1 were observed. Although there was evidence of interhorse heterogeneity in relation to the magnitude of expression of individual genes at each timepoint, within each horse, several inflammatory related genes (e.g. chemokine ligands, interferons and NFKB) were expressed to a higher degree at T0, compared to T1 **(Figure 3.4.7)**. This was evident despite the relatively poor annotation of the current horse microarray. This may reflect relative downregulation of these genes during the training period, consistent with an immunosuppressive effect of exercise, as previously suggested (Robson et al., 2003, Raidal et al., 2001, Raidal et al., 2000).

Genes associated in the different clusters were explored for GO annotation analysis (Biological processes) by DAVID software. A gene list of 76 annotated genes was created from the clusters of genes with higher expression at T0 (Cluster 2, 5, 16, 21, 39, **Appendix IV, S1**). This group included genes involved in cell activation and immune system processes **Table 3.4.3**. The annotated genes upregulated at timepoint T1 are shown in **Table 3.4.4 (full list in Appendix IV, S2)** and are genes mainly related to cellular metabolic processes and to regulation of biological processes.

Clusters 9 and 25 **(Figure 3.4.8)** are representative of a number of clusters that produce the horse-to-horse variation seen also in the PCA and it shows that some horses have higher expression of a group of genes compared to others.

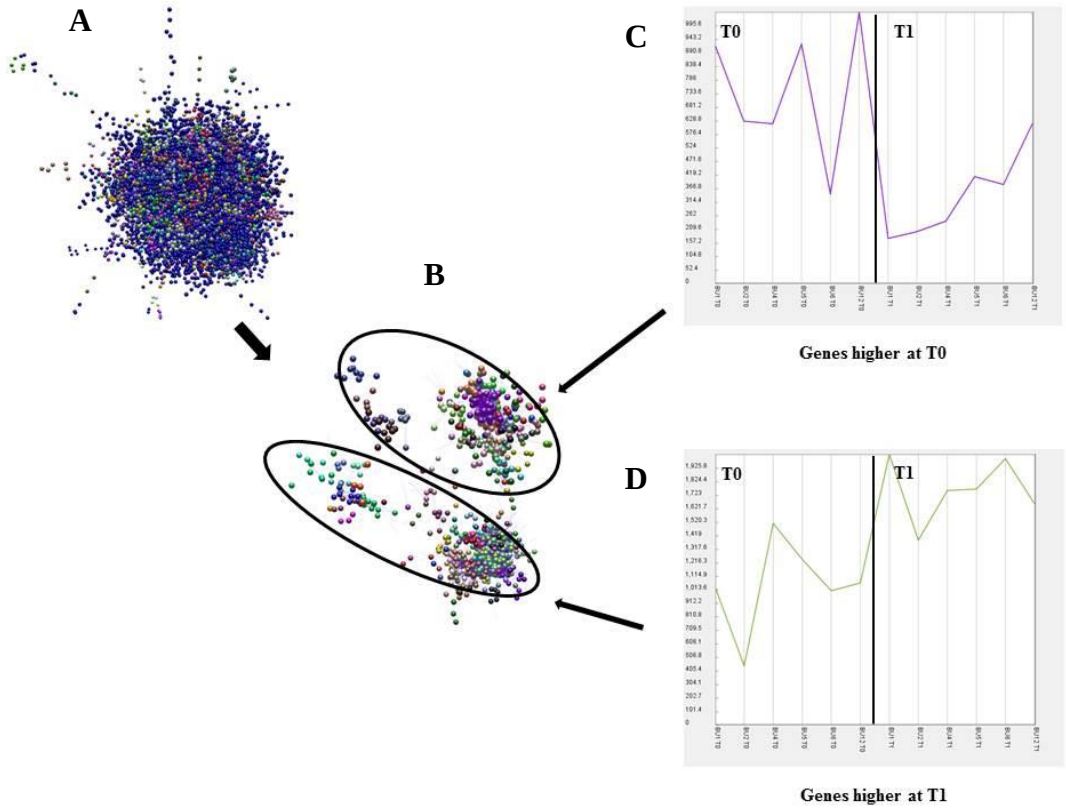


Figure 3.4.6: Network graph analysis of the response of equine AMs to training using Biolayout Express^{3D}.

*The network graph **(A)** represents the gene expression of AMs from 6 racehorses at rest (T0) and during the training period (T1). Genes with a similar expression profile are clustered together. Image **B** shows just the nodes involved in the clusters with an expression profile shown in **C** and **D**. Graphs **(C)** and **(D)** show the pattern of the clusters of genes that were detected higher at timepoint T0 and T1, respectively. The x-axis represents the 6 different horses before (T0) and after (T1) the training period and the y-axis the normalised expression level based on microarray intensity. Horses BU1, BU2, BU4, BU5, BU6 and BU12, as depicted in order (left to right) on the x-axes were included in this analysis.*

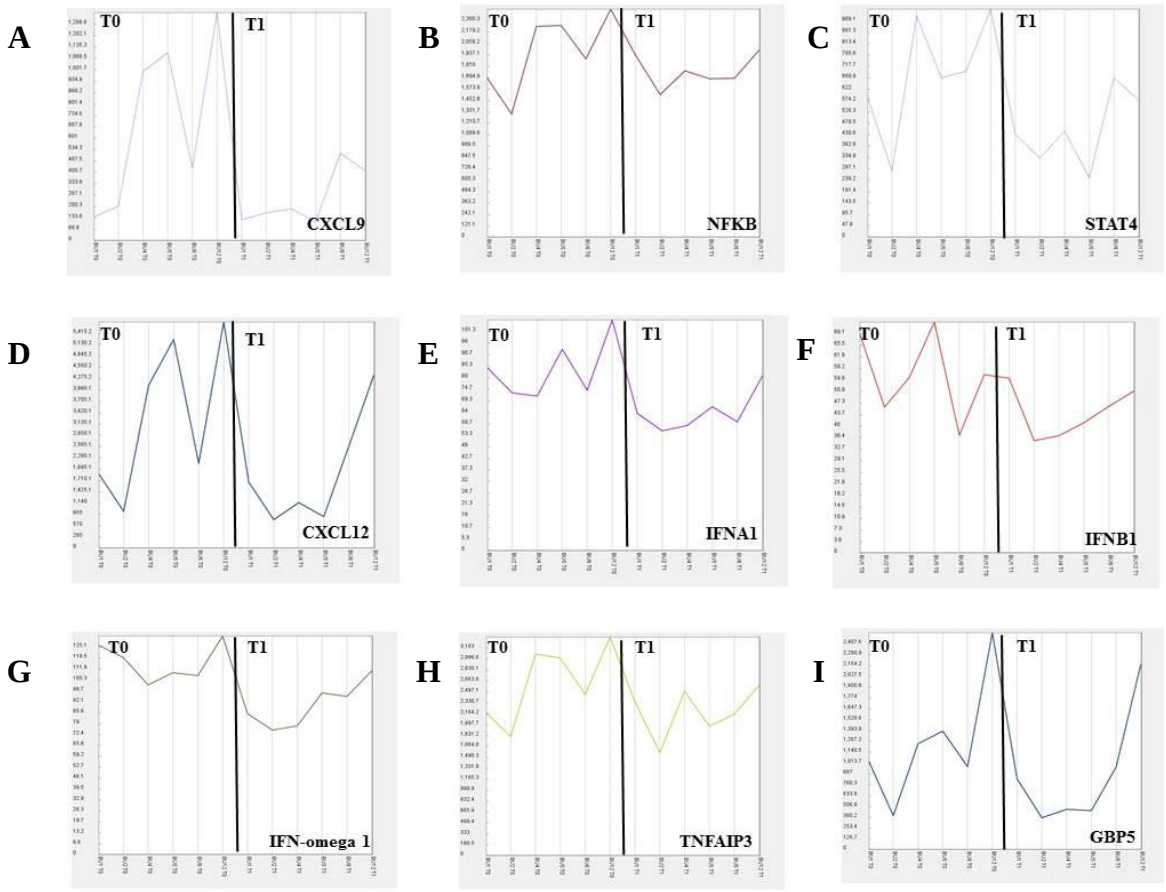


Figure 3.4.7: Expression profiles of immune related genes.

Selection of genes presented here were downregulated during training. These include genes which were either (a) located within the top 5 clusters of genes with higher expression in AMs (Clusters 2, 5,16, 21 and 39) or (b) located within other smaller clusters, yet with a similar expression profile and considered to have potential biological significance (e.g.CXCL9, STAT4, CXCL12). The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse . Horses BU1, BU2, BU4, BU5, BU6 and BU12, as depicted in order (left to right) on the x-axes were included in this analysis. CXCL9, chemokine (C-X-C motif) ligand 9; NFKB, nuclear factor kappa-light-chain-enhancer of activated B cells; STAT4, signal transducer and activator of transcription 4; CXCL12, chemokine (C-X-C motif) ligand 12; IFNA1, interferon alpha 1; IFNB1, interferon beta 1; IFN-omega 1, interferon omega 1; TNFAIP3, tumor necrosis factor alpha-induced protein 3; GBP5, guanylate binding protein 5.

Annotation cluster	Biological process	Enrichment score	Gene count
1	Response to stimulus	7.92	50-58
2	Immune system process	2.7	11-17
3	Immune system development	2.68	3-17
4	Immune response activating cell surface receptor signalling pathway	1.89	3-27
5	Cell development- differentiation	1.26	8-46
6	Response to virus/biotic stimulus	1.04	4-8

Table 3.4.3: The top six annotation clusters associated with T0.

The table shows the top 6 out of 25 annotation clusters created by DAVID software that were related with the list of 117 genes found higher at T0. The enrichment score is a modified Fisher exact p value calculated by the software and the higher it is the more enriched the cluster is. Each annotation cluster contains similar biological subcategories (http://david.abcc.ncifcrf.gov/content.jsp?file=functional_annotation.html). Each biological subcategory contains a specific number of genes; therefore, it is possible for a group of differentially expressed genes to be related to a particular annotation cluster via one or more of these biological sub categories. The range of gene counts (right column) incorporates the different gene counts relating to each biological subcategory.

Gene symbol	Gene name	Cluster
HEATR1	HEAT repeat containing	40
GLRX3	Glutaredoxin 3	40
LYAR	Cell growth-regulating nucleolar protein	40
KIAA0020	Minor histocompatibility antigen HA-8	40
PDCL3	Phosducin-like 3	40
TMEM165	Transmembrane protein 165	40
TOMM70A	Translocase of outer mitochondrial membrane 70 A	40
NOL10	Nucleolar protein 10	40
NOM1	Nucleolar protein with MIF4G domain 1	40
RARS	Arginyl-tRNA synthetase	40
TACC3	Transforming, acidic coiled-coil containing protein 3	40
URB1	Ribosome biogenesis 1	40
CCDC122	Coiled-coil domain containing 122	42
ESM1	Endothelial cell-specific molecule 1	42
OR5B2	Olfactory receptor, family 5, subfamily B, member 2	42
DOT1L	DOT1-like, histone H3 methyltransferase	90
EXTL3	Exostoses (multiple)-like 3	90
MNAT1	CDK-activating kinase assembly factor MAT1	90
HIVEP3	Human immunodeficiency virus type I enhancer binding protein 3	90
PCGF6	polycomb group ring finger 6	90
RBM17	RNA binding motif protein 17	90

Table 3.4.4: Genes with higher expression at timepoint T1.

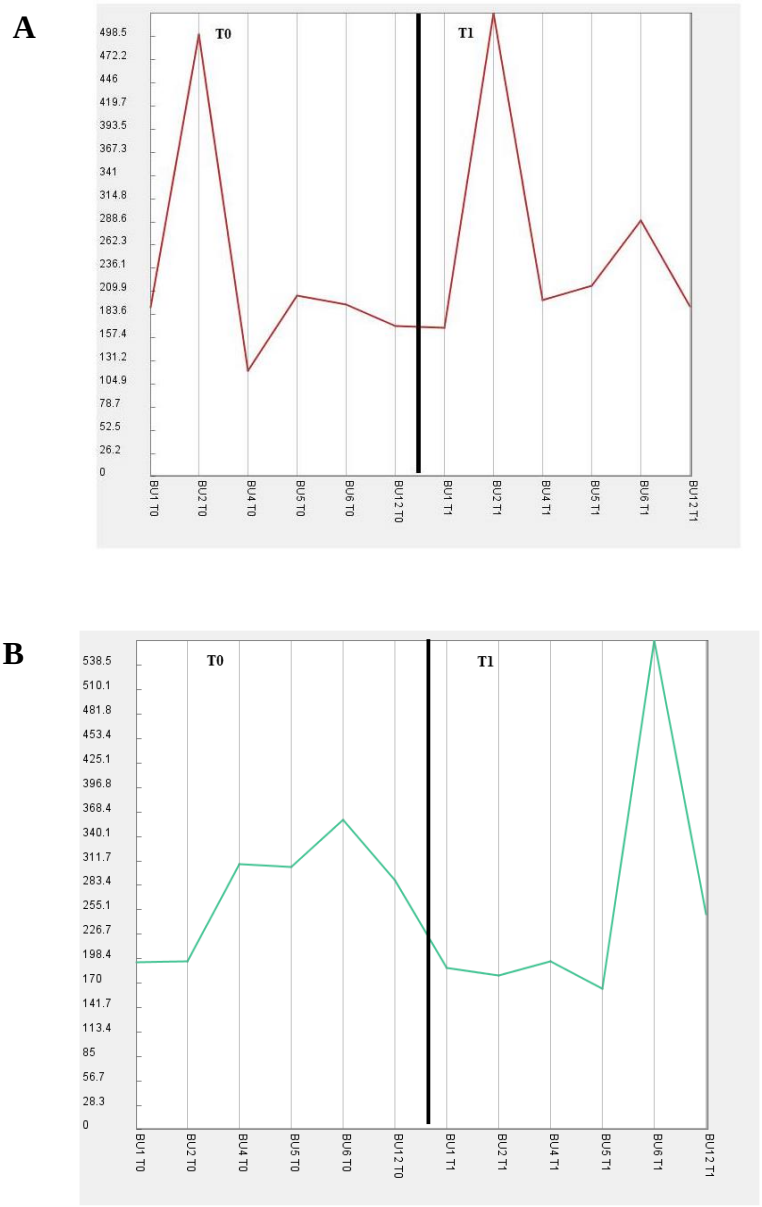


Figure 3.4.8: Expression pattern of clusters 9 (A) and 25 (B).

Horses BU2 ***(A)*** *and Horse BU6* ***(B)*** *had higher expression of all the genes in this cluster than any other horse at either T0 or T1. Horse BU6 was the only horse with upregulated genes in this cluster at T1. Other clusters were also found where individual horses had a unique expression pattern not shared by any other horse. The x-axis shows each horse and the y-axis shows the normalised relative intensity of expression in that horse.*

Chapter 4: Discussion

The principle focus of this thesis was to improve our understanding of equine AMs. Since these are the main cells responsible for bacterial and particulate clearance within the lung, it was hypothesised that they may represent an appropriate therapeutic target cell for IAD, which has been recently defined as a nonspecific inflammatory condition of the equine lower airways. In light of limited published data on equine macrophage biology at the commencement of the study, efforts were directed towards improving knowledge of basic equine AM biology, a necessary prerequisite to addressing the initial hypothesis. The work was greatly facilitated by the abundant yield of macrophages **(Section 3.1.1)** which could be isolated from the airways,peritoneal cavity and the peripheral blood of horses, with cell retrieval numbers several orders of magnitude greater than those achievable from rodent models and differential cytological profiles comparable to those reported from live horses (Richard et al., 2010b, Hawkins et al., 1998). Given the successful cryopreservation process **(Section 3.1.2)**, a single horse could feasibly provide sufficient cellular material for numerous comparative functional studies.

By comparison to other species such as humans (Ratjen et al., 1994), *Rhesus macaques* (Cai et al., 2014), rodents (Sunil et al., 2007, Okamoto et al., 2003), dogs (Mercier et al., 2011), and cows (Jian et al., 1995), equine BALF contained a higher percentage of lymphocytes and an almost negligible percentage of granulocytes **(Section 3.1.3)**. In contrast, equine PLF contained a significant proportion of neutrophils, as previously reported for healthy cattle (Wittek et al., 2010, Oehme, 1969), but not for humans or mice (Kubicka et al., 1989, Ramalingam et al., 2003). Therefore, this equine (and bovine) PLF neutrophilia, which is markedly higher than of the lung compartment, likely reflects species-specific microenvironmental differences within the peritoneal cavity. The mechanism underlying neutrophil recruitment to this site in these species remains undetermined; however, it is most likely related to the presence of low-grade inflammation in the equine peritoneal cavity. Although Barton and Collatos (1999) detected TNF, IL6 and endotoxin only in the PLF of horses with gastrointestinal disease and not in healthy horses, it is possible that the limited sensitivity of the analytical techniques used could result in

failure to detect low concentrations of biologically active analytes, such as endotoxin in healthy horses (Barton and Collatos, 1999).

The studies in **Section 3.1.5** aimed to optimize conditions for the study of equine macrophage responses in vitro. LPS-induced TNFα production was greater when cells cultured in medium containing heat inactivated HS compared to FCS. Others have reported conflicting results, showing a direct stimulatory effect of FCS on equine macrophages, suggesting that a serum derived factor augmented the response to LPS (Figueiredo et al., 2008). Although LBP seems the most likely candidate, the serum content of this protein should be markedly reduced by heat treatment (Figueiredo et al., 2008) and the identification of other potentially responsible serum components may be warranted, particularly as HS is extensively used in cell culture experiments involving macrophages/monocytes derived from a variety of species, including horse (Werners et al., 2004, Ainsworth et al., 2003a, Bryant et al., 2007, Hondalus et al., 1992), human (Kurihara et al., 1990, Collman et al., 1989) and mouse (Orikasa et al., 1993). HS may contain higher levels of albumin, total proteins and endotoxin compared to FCS (Sugimoto et al., 1990). It may also contain CSF1. Active CSF1 protein is absent from FCS, in as much as most bioassays for CSF1 are carried out in FCS-containing medium, but CSF1 is detectable in adult serum in other species and promotes monocyte maturation (Hume and MacDonald, 2012). Nevertheless, consistent with previous findings (Frellstedt et al., 2014), equine macrophages or monocytes were not apparently activated by the cell culture media supplemented with HS or FCS in the absence of LPS.

Endotoxin tolerance, a protective mechanism induced by continuous exposure to low levels of LPS in both human and murine monocytes and macrophages (del Fresno et al., 2009, Dobrovolskaia et al., 2003, Medvedev et al., 2000, Biswas and Lopez-Collazo, 2009), was demonstrated for the first time in both equine AMs and blood monocytes **(Section 3.1.8)**. Indeed this phenomenon may explain the greater sensitivity of equine monocytes, compared with AMs, to LPS stimulation (Grunig et al., 1991), potentially reflecting a level of desensitization of AMs by chronic low level *in vivo* stimulation. Moreover, the levels of TNFα and IL10 released by the

monocytes in this study are consistent with those reported previously in relation to equine PBMCs (Vendrig et al., 2013) **(Figures 3.1.16** and **3.1.17)**. Interestingly, Barton *et al* (1996) reported reduced LPS responsiveness in equine PMs derived from horses with acute gastrointestinal disease, many of which were endotoxaemic (Barton et al., 1996), as previously reported for murine-derived PMs (Medvedev et al., 2000).

4.1 Equine monocytes can differentiate to macrophages *in vitro*

Equine blood monocytes were successfully differentiated to macrophages by rhCSF1 **(Section 3.1.9)**. This finding was not unexpected, since rhCSF1 was previously reported to cause differentiation of human and mouse monocytes (Hume et al., 1987, Warren and Vogel, 1985, Hume and Gordon, 1983) and pig bone marrow cells, (Kapetanovic et al., 2012). There had been limited prior evidence that rhCSF1 was active on equine cells, including the successful differentiation and function of human osteoclasts on equine spongy bone (Perrotti et al., 2009) and osteoclast like cells from equine bone marrow cells (Gray et al., 2002). Similarly, the equine monocyte differentiation achieved by culture in high concentrations of HS was also a novel finding. HS activity on proliferation and differentiation of several primate cell types including human blood monocytes has been repeatedly reported (Patel et al., 1989, Mergenthaler et al., 1988, Potter et al., 1981, Zuckerman et al., 1979). Similarly, incubation with 10% HS resulted in successful differentiation of murine blood monocytes to macrophages (Wirth et al., 1982). Moreover, the presence of HS but not FCS generated granulocyte/macrophage colony formation from murine bone marrow cells (Sugimoto et al., 1990) and represented a prerequisite for murine stem cell differentiation and maintenance of long term proliferation *in vitro* (Dexter et al., 1977). As noted above, CSF1 is at least one candidate protein responsible for this response, but CSF2 produced by the lung (Trapnell et al., 2009) could also contribute.

4.2 Microenvironment significantly influences function and phenotype of AMs and PMs

Consistent with the results derived from studies on other species, equine AMs showed all the characteristics of typical macrophages, including CD14, CD163 and TLR4 expression **(Figure 3.2.5)**, significant LPS-induced TNFα production **(Figure 3.1.7)** and demonstrable phagocytic activity **(Figure 2.3.8)**. Compared to other macrophage populations, AMs have a distinctive profile, likely reflective of their unique microenvironment (Gonzalez-Juarrero and Orme, 2001, Guth et al., 2009). AMs were clearly very different from PMs in the current study, in that the latter cells failed to produce TNFα in response to stimulation with LPS, Poly IC and heat killed *S. typhimurium* **(Figures 3.1.7 and 3.2.1)**. Another study reported LPS-induced TNFα production by equine PMs, but there was marked horse-to-horse variation (Barton et al., 1996). Furthermore, Barton *et al* (1996) relied on measuring TNFα bioactivity. Other studies which have employed the same TNFα bioassay in the assessment of LPS responsiveness in equine PMs found the response to be neither dose nor time dependent (Morris et al., 1990, Hawkins et al., 1998, Morris et al., 1991). Finally, the previous studies on the LPS-responsiveness of equine PMs utilized cells harvested from live horses. Collection of peritoneal fluid from live horses is conducted on standing subjects, whereby a cannula is inserted through the ventral abdominal midline at the most dependent site and the fluid collected represents that which has accumulated at this site by gravity (Morris et al., 1990, Hawkins et al., 1998, Morris et al., 1991). In contrast, the collection procedure used in the current study, which involved high volume lavage of the entire abdominal cavity, may have resulted in the isolation of more representative population of cells including those loosely adherent to the peritoneum.

The failure of PMs to release TNFα protein, as measured by ELISA, was further supported by the lack of TNFα mRNA detection by RT qPCR **(Figure 3.2.4)**. These combined data are consistent with the results of several studies which have shown relatively low levels of LPS-induced TNFα production by mice PMs (Bradbury and Moreno, 1993, Zheng and Specter, 1996, Sherry et al., 2007, Skuladottir et al., 2007). The biological reason for this apparent deficiency in responsiveness of equine PMs is

unclear but may reflect a degree of LPS tolerance. Although the PM harvesting procedure was not conducted in a completely aseptic manner, it is unlikely that PM stimulation occurred during this process due to the magnitude of dilution with the lavage fluid (6L) and the immediate post sampling separation, isolation and exposure of the cells to antibiotic containing medium. None of the horses sampled had any evidence of gastrointestinal disease, which may have permitted significant endotoxin translocation across the intestinal wall prior to euthanasia. Therefore, low level endotoxin translocation may routinely occur in this species and could also explain the relatively high ratio of neutrophils within the peritoneal cavity of even healthy horses.

There are reports that argue against an "ante-mortem" endotoxin tolerance hypothesis. Firstly, endotoxin was reported to be undetectable in the peritoneal fluidof healthy horses, rather it was detectable only in horses with colic (Barton and Collatos, 1999). Secondly, endotoxin tolerance in murine PMs has been associated with downregulation of TLR4 expression (Nomura et al., 2000). The equine PMs showed low surface expression of CD14, rather than TLR4, a finding also reported in rabbit AMs exposed to LPS or *E.coli in vivo* or *in vitro* (Lin et al., 2004). Considering the involvement of CD14 in the recognition and phagocytosis of gram negative bacteria (Grunwald et al., 1996), this lack of CD14 expression on the equine PMs also explains their poor phagocytic capacity compared to AMs **(Figure 3.2.8)** a phenomenon also reported in rodents (Changqi et al., 2013, Gjomarkaj et al., 1999).

Another possible mechanism of endotoxin insensitivity is the production of IL10, an anti-inflammatory cytokine that is well documented to inhibit the LPS-induced production of pro-inflammatory cytokines, such as IL1, TNFα, IL6 and $PGE_{2,}$ by macrophage cell lines and peritoneal macrophages, with a particular effect on TNFα (Fiorentino et al., 1991). Furthermore, the presence of human IL10 significantly reduced the level of inflammatory mediators, such as IL6, TNF and $PGE_{2,}$ by equine PMs, with a particular effect on TNFα, following LPS treatment (Hawkins et al., 1998). In the current study, LPS-stimulated AMs and PMs produced very low levels of IL10, even at a 24h time-point **(Figures 3.1.8**, **3.1.10** and **3.1.11)**. This lack of LPS induced IL10 production by AMs has also been reported in human- (Thomassen

et al., 1996), murine- (Salez et al., 2000) and pig-derived cells (Liu et al., 2012) and although the underlying mechanisms for this remain unclear, it has been proposed that regulation of its secretion may be influenced by certain undetermined environmental factors, such as surfactants (Salez et al., 2000, Wright, 1997). TLR activation of murine AMs and PMs was shown to inhibit IL10 signal transduction and IL10 induced immunosuppression at the level of TNFα (Fernandez et al., 2004). The secretion of IL10 by AMs is enhanced in response to excessive inflammation. Laan *et al* (2006) reported greater LPS-induced *IL10* production from AMs harvested from horses with pre-existing airway inflammation, compared to healthy horses (Laan et al., 2006).

Differences between AMs and PMs were further supported by gene expression arrays **(Section 3.2.6)**, as seen in other species (Freeman et al., 2012, Hume et al., 2010, Changqi et al., 2013) (www.biogps.gnf.org) . Both PCA **(Figure 3.2.9)** and network analysis **(Figure 3.2.12)** revealed a clear distinction between the two cell populations. AMs showed greater expression of genes related to macrophage differentiation and development, such as CD14, CD68, CD71, CD163 and CSF1 and its receptor (**Figure 3.2.13)** (Hume et al., 2010, Akagawa et al., 2006, Reynier et al., 2012), data which complemented the flow cytometry results. The *CS2RB* gene detected in this group of genes has previously been shown in humans to regulate surfactant homeostasis, with recessive mutation leading to pulmonary alveolar proteinosis (Suzuki et al., 2011). Moreover, AMs showed relatively greater expression of *FOXP1,* which is known to play a key role in lung development, mainly in lung epithelium (Shu et al., 2007). Consistent with their high capacity for phagocytosis compared with PMs, AMs showed relatively greater expression of several members of the scavenger receptors family, c type lectins and others, shown to be also essential for functions such as endocytosis, phagocytosis, adhesion and antigen recognition (*CD14, CD40, CD48, CD68, CD163, CD276, CLEC2B, MRS1*) **(Figures 3.2.13** , **3.2.14** and **Appendix II, S3)** (PrabhuDas et al., 2014).

Equine AMs also showed relatively greater expression of certain important pattern recognition receptors, including *TLR3,* which recognises viral double stranded RNA, *TLR6,* which recognises bacterial lipopeptides and mycoplasma and both *TLR7* and

TLR8, which recognise RNA from RNA viruses (Kumar et al., 2011). Even though flow cytometric analysis revealed a slightly greater cell surface expression of *TLR4* on AMs compared to PMs **(Figure 3.2.6),** its gene expression was not detected by analysis of the microarray data as being statistically significantly greater in this cell population. However, this apparent inconsistency can likely be attributed to the thresholds applied in the analysis (e.g. Pearsons correlation, fold change and p-value) **(Section 2.10)**, since focused analysis revealed the gene expression intensity of this receptor to be slightly greater in AMs compared to PMs (*data not shown*). Other studies have also confirmed TLR4 mRNA expression on horse AMs (Waldschmidt et al., 2013, Frellstedt et al., 2014).

AMs have been considered to exhibit an M2 or alternatively activated phenotype, to suppress spontaneous inflammation in the lung (Jones et al., 2013). Several genes related to this state of polarisation were more highly expressed in AMs **(Figure 3.2.13 and 3.2.14)**. These included *TGFB1*, which plays a key role in alveolar homeostasis (Lambrecht, 2006), *CSF1*, which promotes this alternative state of activation (Jones et al., 2013) and the C type lectin *MRC1*, also shown to be highly expressed in porcine AMs along with several other C type lectins (Freeman et al., 2012, Kapetanovic et al., 2013). The increased expression of *MAP3K2* in equine AMs, as detected in the clusters, was also found in porcine AMs compared to bone marrow or monocyte derived macrophages **(Figure 3.2.14)** (Kapetanovic et al., 2013).

By contrast, PMs exhibited a gene expression profile that supported the view that their unresponsiveness to LPS was due to previous exposure to an activating stimulus. In particular, numerous pro-inflammatory as well as anti-inflammatory cytokines were detected in the gene clusters more highly expressed in PMs **(Figure 3.2.15, Appendix II, S4)**. No defined polarisation could be attributed to the PMs, as highly expressed transcripts were identified which could be related to both classical [M1] (*CXCL1*, *CXCL3*, *IL1A*, *IL1B*, *IL8*, *IL23A*, *NFKB2*) and alternative [M2] (*CCL22*, *IL4R*, *IL10*) macrophage activation. This finding is consistent with both mouse and human dataset analyses, which have also failed to support the existence of a transcriptional specific outline linked to the M1 or M2 activation status (Wells et

al., 2003, Mabbott et al., 2010). A novel hybrid polarisation has recently been reported in murine macrophages following the induction of LPS tolerance (O'Carroll et al., 2014). Interestingly, certain transcripts of this profile were also detected in equine PMs (*IFNA2*, *IL6*, *THBS1*). In addition, the equine PM expression profile showed characteristics previously attributed to both wound healing (*CCL22*, *IL4R*, *MMP1*, *MMP14*) and regulatory macrophages (high *IL10*, low *IL12* expression).

The relatively greater expression of several neutrophil chemoattractants, including *CXCL1*, *CXCL3*, *CXCL6*, *IL8* and *CSF3* and its receptor **(Figures 3.2.15 and 3.2.16)** could contribute to the presence of neutrophils within the PLF. Furthermore, the hypothesised role for low grade endotoxin translocation in this neutrophil recruitment may be supported both by the expression of TLR4 on the equine PMs and the reported TLR4-dependent increase in *CXCL1/2* expression in murine PMs and peritoneal mast cells) following LPS stimulation, with resultant neutrophil recruitment to the peritoneum (De Filippo et al., 2013).

In contrast to AMs, PMs reside within a hypoxic milieu and consistent with this microenvironment, some hypoxic induced genes were found to be more highly expressed in PMs, including *VEGFA* and its receptor *FLT1*, *CXCR7* and *HIF3A* **(Figures 3.2.16, Appendix II, S4)**. Previous studies have shown that such a hypoxic environment can result in the induction of genes involved in the regulation of various cell functions such as angiogenesis, metabolism, cell survival and apoptosis (Ando et al., 2013, Roda et al., 2011, J. Martin, 2013). Similarly, transcript *FGF2* related to wound healing and development of epithelial and endothelial layers was also highly expressed in PMs.

Compared with PMs, AMs are continuously challenged on a breath-by- breath basis with inhaled air containing a variety of pro-inflammatory agents and significant reliance is placed on this cell population to cope with this challenge in the most appropriate manner. The greater LPS responsiveness and phagocytic capacity of AMs compared to PMs probably reflect their adaptation to this key role, while minimizing the likelihood of collateral tissue damage, which may result in tissue remodeling and impaired gas exchange. Despite the constant pro-inflammatory

challenge of the airways, the negligible neutrophil presence within this compartment is reflective of the efficiency with which the AM fulfills this role.

4.3 The response of equine AMs to LPS

The equine AM response to LPS stimulation has been studied by others (Grunig et al., 1991, Waldschmidt et al., 2013, Schneberger et al., 2009, Laan et al., 2005), but the current study was the first to investigate the resultant alteration in genome wide transcriptional profile. A core set of inflammation related genes were upregulated 6h after LPS stimulation **(Figures 3.3.5 and 3.3.8. A-L)**. Many of these genes have previously been reported to be upregulated in LPS treated AMs from a variety of species, including the horse (Waldschmidt et al., 2013, Liu et al., 2012) and represent part of a stereotypical immune response to endotoxin (Boldrick et al., 2002, Wells et al., 2003). In particular, known targets of both MyD88 (*TNF*, *IL1*, *IL6*, *IL10*) and TRIF (*IFNB*, *CCL5* commonly known as *RANTES*) pathways were activated by LPS. This finding contrasts with another horse study, where equine monocytes seem to activate mainly the MyD88 pathway following LPS treatment (Figueiredo et al., 2009). This discrepancy potentially indicates another cell specific difference. Further whole transcriptomic studies comparing these two cell types would help clarify this issue.

Additionally, LPS stimulation also induced the expression of mRNA for various mediators of inflammation suppression, including the following: IL1RN, which inhibits IL1A and IL1B production; SOCS3, a feedback regulator of classical macrophage activation (M1) (Spence et al., 2013) **(Figures 3.3.5 and 3.3.8. M-N, Appendix III, S2)**. LPS-induced *SOCS3* expression has previously been demonstrated in AMs, where it was shown to subsequently suppress LPS-induced pro-inflammatory gene expression, a property considered to minimize excessive inflammation which may lead to preventing collateral tissue damage in the lung. Consistent with this role, *SOCS3* depletion in murine AMs resulted in excessive secretion of inflammatory cytokines and chemokines by AMs (Spence et al., 2013).

In agreement with the results of previous studies on murine, human and porcine-derived cells (Wells et al., 2005, Joshi et al., 2003, Kapetanovic et al., 2013, Reynier et al., 2012), LPS stimulation also resulted in the upregulated expression of genes related to programmed cell death and apoptosis (*CASP4*, *CASP7*, *CASP8* and *APAF1*), a process with an ongoing interactive association with inflammation and a key regulator of tissue homeostasis (Joshi et al., 2003). Considering the continuous exposure of AMs to pro-inflammatory agents, recruitment of processes such as inflammation suppression and apoptosis likely play a key role in preventing excessive inflammation and limiting collateral tissue damage *in vivo*. The response to LPS is a cascade of induction of transcription factors, which in turn induces further downstream targets. In this study, those factors include *STAT4*, *ETV6*, *ETV7*, *GATA6* and *BATF3*. Additionally, LPS-induced expression of several growth factors and some of their receptors was also detected. A main biological role attributed to CSF3 is neutrophil chemoattraction (Roberts, 2005). Moreover, CSF2 is responsible not only for the maturation and proliferation of granulocytes and monocytes/macrophages but also for the cytoprotection of AECs (Standiford et al., 2012), potentially representing a further mechanism by which excessive tissue damage is limited. CSF2 signaling via its receptor delays apoptosis of AECs and plays an essential role both in surfactant homeostasis (Suzuki et al., 2011) and pro-inflammatory cytokine secretion in response to LPS via PU.1, which regulates the TLR4 signaling pathway (Berclaz et al., 2007, Standiford et al., 2012). Furthermore, LPS stimulation also induced increased expression of several guanine nucleotide exchange factors and guanine nucleotide binding proteins (*G proteins*) which are known to interact with CD14 to promote LPS signaling via p38MAPK phosphorylation (Solomon et al., 1998). Interestingly, the phenomenon of significant inter-individual differences in response to LPS, observed here in both horse and human subjects **(Figure 3.3.2 and 3.3.11, respectively)**, has been reported before in equine (Werners et al., 2006), human (Michel et al., 2003) and murine studies(Coutinho et al., 1977) suggesting a diverse responsiveness/susceptibility to bacterial infections in different individuals .

A comparative microarray study of LPS stimulated human and murine macrophages found that 24% of the orthologue genes between the two species were differently regulated (Schroder et al., 2012). These included *CCL20, CXCL13, IL7R, P2RX7, IDO* and *STAT4*, all of which were induced only in human, and not murine LPS treated macrophages. Although these data may partly reflect cell specific differences, as they were mainly derived from murine bone marrow and human blood monocyte-derived macrophages, subsequent work has also shown the differential expression of these human-induced genes in both porcine bone marrow and blood monocyte-derived macrophages (Schroder et al., 2012, Kapetanovic et al., 2012), data which are also supportive of species-specific differences. Comparison of the equine AM LPS-induced gene expression with the data reported by Schroder *et al* (2012) revealed the expression of some of the human specific genes by equine AMs. These included the following: *IL7R*, which is essential for T and B lymphocyte development and has variants associated with inflammatory diseases (Gregory et al., 2007, Kondo et al., 1997); *STAT4*, which is essential for the induction of IL12 signaling, involved in the Th1 response and an integral part of the host immune response to gram negative bacteria in the lungs (Deng et al., 2004); *XAF1*, a negative regulator of the inhibition of apoptosis (Liston et al., 2001) and others (*BATF3, GPR183, RBMS2, RAPGEF2, PARP9, KYNU* and *IDO*) **(Figures 3.3.13 and 3.3.14)**. Other LPS-induced human specific genes, such as *CLL20* or *CXCL13*, were not included in the Affymetrix arrays used in this study. Among the total differentially expressed LPS-induced genes in the present study, 66 orthologues were detected between the human and horse **(Figure 3.3.13)**. Sixty three of these followed a similar expression profile in the two species, including both human specific genes and genes involved with the stereotypical immune response to bacteria. A few, such as *IL6* and *ISG20*, showed a marked difference in their fold change, but the studies are not directly comparable, since Reynier *et al* (2012) harvested the cells following LPS stimulation *in vivo*. *EREG*, involved in the cell cycle and *RGS2*, a regulator of G protein signaling, were induced only in human AMs; whereas *RDH10* was expressed only in equine AMs (Bauer et al., 2009). *TLR3*, a receptor generally associated with viral infection, was induced in equine, but not human-derived macrophages, and has

previously been reported in murine-derived macrophages (Heinz et al., 2003). Whether these differences are functionally important remains to be determined.

4.3.1 Arginine and tryptophan metabolism differs across species

The production of NO from metabolism of arginine is a key component of the response of rodents to pathogen challenge that is not conserved in human macrophages. The differences are associated with complete lack of sequence conservation in the promoters and enhancers of the iNOS locus between rodents and humans (Thoma-Uszynski et al., 2001, Geller et al., 2006, Mellott et al., 2001, Yu et al., 2005, Schroder et al., 2012). The current study suggested that horse macrophages resemble those of humans. The absence of NO metabolism in response to LPS stimulation in AMs **(Section 3.17)** contrasts an earlier report on AMs (Hammond et al., 1999a) and another one on equine bone marrow-derived macrophage cell line (e-CAS) (Werners et al., 2004, Bryant et al., 2007), but is consistent with two other equine studies on AMs (Johnson et al., 1997) and PMs (Hawkins et al., 1998). Pig macrophages also fail to induce iNOS in response to LPS (Kapetanovic et al., 2012). As in the pig, LPS did not induce any of the genes involved in the NO pathway in equine AMs **(Appendix III, S1 and S2)**. The similarity in response of the equine and human-derived cells is supported by the high level of conservation between the human and horse promoters and the poor alignment of the promoter sequences between mouse/horse and mouse/human **(Figure 3.1.14)**.

Although NO has been detected in BALF collected from horses, its production has been attributed to bronchial epithelial cells (Costa et al., 2001), in which the expression of iNOS was reported to increase in horses with summer pasture-associated obstructive pulmonary disease, a lower airway inflammatory disorder that resembles asthma (Costa et al., 2001). Similarly, in light of the accepted lack of NO production by human macrophages in culture (Schneemann et al., 1993), the detection of NO in human BALF and exhaled gas has been attributed mainly to alveolar epithelial cells (Hamid and Springall, 1993). Indeed, increased production

has been reported in the epithelial cells of asthmatic patients (Hamid and Springall, 1993). An alternative pathway for NO production has been reported, whereby nitrite and nitrate can reform NO independently of iNOS, mainly under hypoxic conditions (Lundberg et al., 2008). If this pathway exists in the horse, it may contribute to the reported NO detection in BALF.

Whilst equine macrophages do not apparently metabolise arginine, they share with humans the inducible metabolism of tryptophan. All of the components of this pathway are induced by LPS in both human (Schroder et al., 2012) and pig (Kapetanovic et al., 2012) macrophages. Indeed, IDO activation is involved in many human diseases including endotoxaemia and sepsis (Lögters et al., 2009, Campbell et al., 2014). The detection (RT qPCR and microarray) of LPS-induced upregulation of IDO in equine AMs complements the findings of previous studies which reported IDO upregulation in horse AMs during intracellular bacterial infection (Heller et al., 2010). Induction of *KYNU*, which encodes an enzyme involved in the IDO pathway, has been proposed as a potential early marker for sepsis in patients with severe trauma (Lögters et al., 2009). Despite the importance of this pathway in human inflammation and the recognized absence of *IDO* gene upregulation in murine macrophages, the mouse continues to be commonly used as a model for human inflammatory research. Such fundamental differences in cellular biology continue to support the development and use of other more appropriate animals, such as pigs, as models for human inflammatory diseases (Fairbairn et al., 2011, Kapetanovic et al., 2012). Although the results of the current study largely supported inter-species conservation of the LPS responsiveness of the AM at the level of gene expression, they also highlighted important specific similarities between equine and human macrophage biology, thus potentially supporting the use of the horse as an appropriate model for human inflammatory research.

4.4 The effect of training on equine AMs

As previously reported (Raidal et al., 2000, Ainsworth et al., 2003a), the studies in **Section 3.4** did not produce much evidence of training-associated changes in total

number of recovered BALF cells and their DCC. In agreement with the results of previous studies, training also did not appear to affect the morphological appearance of AMs (Huston et al., 1986, Adamson and Slocombe, 1995) or their LPS responsiveness, as assessed by TNFα release **(Figure 3.1.7 and 3.4.1)** (Frellstedt et al., 2014). Although the PCA revealed a distinct separation in gene expression between the two time points **(Figure 3.4.5),** it was clear that the magnitude of this training-associated alteration varied significantly between individual horses, an observation which was also clearly demonstrated at the source of variation plots **(Figure 3.4.4)**. Although no clinical abnormalities were detected in these horses prior to their recruitment to the study, the results of subsequent BALF cytological examinations indicated the presence of small airway inflammation in all the horses, potentially supportive of subclinical IAD and/or EIPH (Richard et al., 2010a). Accordingly, a much larger dataset would be required to demonstrate the significance of any impact of training against a substantial background of inter-individual variation.

Although the initial analysis did not reveal any genes with large fold changes related to training, subsequent data analysis by Biolayout *Express*^{3D} software revealed that some immune related genes such as chemokines, the transcription factor *STAT4*, *NFKB* and several *IFNs* were more highly expressed in samples obtained at rest compared to those obtained during training **(Figure 3.4.7)**. These data complement the findings of Frellstedt *et al* (2014), who reported a significant training-associated decrease in the basal expression of *IFNB* in equine AMs. Similar results have also been reported in relation to human PBMCs, where prolonged intense training resulted in reduction of the NFKB signaling pathway, indicating an immunosuppressive effect of training on blood cell compartments (Sousa e Silva et al., 2010). Despite the limited sample size, the analysis supports some direct effect of training on gene expression, particularly of immune-related genes, that may play a role in increasing susceptibility to opportunistic infection and suggest that larger population studies would be more informative.

4.5 Limitations of the study

Despite efforts to optimize the experimental designs and maximize the validity of the data generated, there were inevitable and unavoidable limitations throughout the study which could theoretically have influenced the results. Despite extensive research on equine inflammatory diseases, specific information on the equine mononuclear phagocyte system (MPS) is lacking, a deficiency which can largely be attributed to the limited availability of appropriate molecular reagents. Consequently, many studies have relied on the successful use of cross reactive human/murine reagents, some of which were also used in this study (e.g CD163 and TLR4 antibodies). Availability of a wider range of commercially available horse specific reagents throughout the period of study would likely have provided additional and novel information on equine AM biology. Similarly, the comparatively poor annotation of the equine genome may have resulted in the failure of the microarray experiments to reveal additional and important information. Finally, there are inherent limitations when working with the horse, either as a species of interest or a comparative animal disease model. These include financial constraints in relation to general maintenance of horses and limitations in relation to the adherence to optimal study designs when reliance is placed on the acquisition of samples from client-owned animals, particularly those which are actively competing. The inability to ensure that BALF samples were derived from the racehorses at exactly the same stage of training is an example of such a limitation in relation to this study.

4.6 Conclusions and future perspectives

The data generated throughout this study have provided a platform on which the design of future studies aimed at prophylactically and/or therapeutically targeting the AM, can be based. To date, a limited number of studies aimed at suppressing or activating the equine AM have been conducted, using anti-inflammatory molecules or immunomodulators. An attempt to investigate the immunosuppressive effect of the filaria-derived immunomudulator, ES-62 on equine AMs was performed during

this study; however no promising results were obtained (*data not shown*). Currently, another immunomudulator, iPPOV, presently marketed under the tradename Zylexis® by Zoetis, is under investigation.

The comparative data derived from this study refute the concept that "a macrophage is a macrophage" and highlights the importance of studying cells derived from the tissue of interest. Recent global analyses from mouse, human and pig have highlighted the fact that tissue macrophages from other locations also differ radically in the their gene expression profiles (Hume et al., 2010, Mabbott et al., 2010, Freeman et al., 2012) and there are a few transcriptomic studies highlighting tissue differences in the horse. Graham and coworkers commenced the investigation of the horse transcriptome using human microarrays on the brain, liver and articular chondrocytes (Graham et al., 2010). Other studies using microarrays (Huang et al., 2008b) and RNA-seq (Coleman et al., 2010) investigated the equine transcriptome of different tissues and detected tissue specific expression profiles correlated with specialised tissue functions. These studies were not focussed at the level of tissue macrophage differentiation, although such information can be extracted from the data (Freeman et al., 2012). It will therefore be of significant interest in the future to use enzymic disaggregation to obtain equine macrophage populations from organs such as bone marrow, brain (microglia), spleen, liver (Kupffer cells), and gastrointestinal tract. Moreover, further flow cytometric and gene expression experiments on equine monocyte subpopulations would allow the comparison with human and mouse monocyte subtypes. In each case, the cells could be harvested in large numbers and frozen for future analysis and the results would provide insight into both equine innate immunity and serve as models for humans.

Horses, like other daylight-active animals including humans and pigs, have evolved differently to nocturnal animals, such as the mouse. The comparative analysis of LPS-induced gene expression of equine AMs with that of other species revealed significant similarities with human-derived cells and significant differences with murine-derived cells, supporting the potential suitability of the horse as a model of human lung inflammation and endotoxaemia. However, further investigation of this possibility using Cap analysis gene expression (CAGE) or RNA-seq could be

performed to examine in depth key similarities and differences in the response of equine and human macrophages/monocytes to LPS stimulation. Such findings could provide further insights into the shared phenomenon of high sensitivity of humans and horses to LPS and potentially provide therapeutic targets of endotoxaemia and sepsis in man, an area that has yielded little success through the use of rodent models.

As with many domesticated animals, breeding of horses has produced an array of breeds selected for specific traits. More than 175 horse breeds are defined, and given the nature of line-breeding, each breed is likely to harbour breed-associated naturally occurring mutations which may be associated with enhanced susceptibility or resistance to several diseases. Since many equine diseases share characteristic features with a great range of human diseases, the use of technologies such as next generation sequencing and genome-wide association studies, could facilitate the detection of potential quantitative trait loci or even genes involved in specific diseases. Furthermore, the potential benefits of using genetic studies in horses for comparative purposes includes the extensive equine families available for inclusion.

The genetic predisposition of equine respiratory tract diseases have recently been reviewed (Gerber et al., 2014). RAO, a common equine respiratory disorder and a recognised model for human asthma, dominates the field of interest in relation to the role of genetics on lower respiratory tract disease susceptibility. A few chromosomal regions in the horse genome have already been linked to RAO and several candidate genes of interleukins have been detected in these regions (Jost et al., 2007, Swinburne et al., 2009). *IL4RA* a polymorphic gene involved in Th2 response in asthma was recognised as a major candidate gene for both asthma and RAO susceptibility (Howard et al., 2002). Nonetheless, a few contradictory results have already been published regarding the role of this gene, suggesting loci heterogeneity for such conditions and highlighting the dual effect of genetic background and environmental factors on their development (Jost et al., 2007, Howard et al., 2002, Mújica-López et al., 2002). These findings reflect the complex pathogenesis of both RAO and asthma, which is characterised by a polarised or mixed Th1/Th2 immune response (Ainsworth et al., 2003b, Lavoie et al., 2001, Abdulamir et al., 2008).

Given the clinical and suggested aetiopathogenic similarities between IAD and RAO, it is possible that a common pathologic mechanism exists between these two conditions. Indeed, it has been proposed that IAD may be a precursor of RAO (Ainsworth et al., 2003b, Giguère et al., 2002, Lavoie et al., 2011). Nevertheless, the genetic basis of IAD has been poorly studied, largely because of the inherent difficulties in accurately defining the disease phenotype. Despite some attempts to detect more reliable non-invasive diagnostic markers for IAD, diagnosis is based mainly on the BALF DCC analysis (Couetil et al., 2007, Robinson, 2003). A more robust phenotypic characterisation of the IAD is essential for future genetic studies aimed at identifying genetic predispositions and further elucidating aetiopathogenic pathways with a view to informing novel treatment and management strategies. Moreover, identification of specific genetic susceptibilities to IAD and/or training asoociated immune derangements may permit more selective breeding in the future focussed on reducing disease prevalence

The horse is a natural athlete and findings derived from equine exercise studies could be extrapolated to elite human athletes and *vice versa*. Limited studies in horses have used microarrays to assess the effects of long-term intense exercise on peripheral blood monocyte global gene expression (Barrey et al., 2006, Capomaccio et al., 2010). The application of any translational studies to investigate the effect of intense exercise/training on the function of AMs derived from racehorses seems most appropriate within the field of exercise immunology. Several mechanisms and biofunctions were revealed in this thesis which could be regarded as consistent with a training-associated immunosuppression and increased susceptibility to opportunistic infection. Consequently, the data generated did provide further justification for future longitudinal studies incorporating a larger sample size derived from appropriately phenotyped horses, a more defined and repeatable training regime and additional "post-training" sample time points.

The major limitation throughout the study was the restricted commercial availability of horse-specific reagents. Although horse-specific arrays were used, they remain relatively poorly annotated. Over time, this limitation will become progressively less problematic as the annotation of the horse genome improves. An alternative means of

overcoming this limitation will be the extended use of techniques such as CAGE or RNA-seq. The recent publication of global analyses of human and mouse transcriptomes by the FANTOM5 consortium (www.fantom.gsc.riken.jp/5) and ongoing activities in Roslin to extend sheep, pig and chicken expression atlases based upon CAGE and RNA-seq will provide resources for comparative studies in the horse.

Together, the work described in this thesis has significantly increased our knowledge of equine macrophage biology, particularly that of AMs. Tissue-dependent heterogeneity of macrophage function and phenotype was detected and has formed an appropriate platform of knowledge on which future studies can be based. Moreover, novel and valuable information on the effect of race training on AM gene expression could contribute to the overall knowledge base on the potential mechanisms, which may be involved in promoting susceptibility to exercise-associated pulmonary inflammation, such as equine IAD. The inter-species comparative data have provided support for the potential role of the horse as a suitable model for studies on human macrophage biology. The completion of the equine genome annotation would provide a major tool for future transcriptomic horse studies, the findings of which could be applied to humans.

Chapter 5: Appendices

All Excel files contained in CD on inside back cover.

5.1 Appendix I

Primer Name	Standard curve equation	Pearsons Correlation	Efficiency (%)
TNFα	Y= -3.623 * LOG(X) + 19.99	0.945	99.8
IDO	Y= -3.374 * LOG(X) + 22.55	0.972	97.9
STAT 4	Y= -3.404 * LOG(X) + 21.43	0.960	97.7
IL7R	Y= -3.582 * LOG(X) + 26.81	0.998	90.2
CYP27B1	Y= -3.208 * LOG(X) + 25.61	0.922	105.0
TNIP3	Y= -3.590 * LOG(X) + 24.12	0.928	108.3
18S	Y= -3.607 * LOG(X) + 9.12	0.977	90.3

5.2 Appendix II

S1- Genelist of 451 transcripts differentilly expressed between AMs and PMs

S2 - Putative annoation of equine uncharacterised proteins across the human genome in Enesembl

S3 - Top clusters of genes more highly expressed in AMs

S4 - Top clusters of genes more highly expressed in PMs

5.3 Appendix III

S1- Genelist of LPS treated AMs

S2 - Top five clusters of genes upregulated in AMs after LPS treatment

S3 - Top five clusters of genes downregulated in LPS treated AMs

S4 - Genelist of LPS treated human AMs

5.4 Appendix IV

S1 - Top five clusters of genes more highly expressed at T0

S2 - Top clusters of genes upreglated at T1

Chapter 6: References

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